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(54) Title: FULLY–COATED, UNIFORM–SIZED METALLIC PARTICLES

(57) Abstract

A novel small size (less than 50μ) metallic particle having a metallic core coated by a rigid inorganic surface protection layer is disclosed. A novel superparamagnetic particle comprising a metal oxide core embedded with a preferably homogeneous inorganic seed, and a rigid inorganic surface protection layer to which a wide variety of molecules may be coupled is also disclosed. Uses of the novel metallic particles and methods for their preparation are also disclosed. The novel metallic particles are chemically, biologically and colloidally stable, and are of highly uniform size dispersion. The metallic particles have a wide variety of applications in systems involving separations steps, in particular the separation of biological materials.

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FULLY-COATED, UNIFORM-SIZED METALLIC PARTICLES

FIELD OF THE INVENTION

This invention relates to the production and use of intermediate diameter (between 0.03 and 1.0μ in diameter) metallic particles that are sealed with an inorganic surface protection layer which will prevent the metallic core from interacting with and/or leaching into a solution that bathes the metallic particles.

A preferred embodiment of the invention provides superparamagnetic particles in the indicated size range comprising the surface protection layer of the present invention, and a method for the production of such particles.

Another preferred embodiment of this invention provides a method of producing metallic particles in a monodisperse size range (with a standard deviation of no more than thirty five percent of the mean particle diameter) by forming such metallic particles using a homogeneous seed crystal, then further coating the resulting monodisperse particle with the inorganic silica surface protection layer of the present invention.

Yet another preferred embodiment provides a method of producing the aforementioned monodisperse metallic particles from ferric, zinc, nickel or cobalt oxides such that the resulting monodisperse, encapsulated particle is superparamagnetic.

Such particles have wide application in industrial and biological separation processes including, but not limited to, the removal of toxic compounds from industrial waste streams, the clean-up of industrial and environmental sites, the detection of contaminants in sewage treatment processes (and any other processes which may also involve strong oxidative conditions) and a wide variety of biological applications. The magnetic particles of the invention may be used in variety of applications where separation steps are used for removing or enriching certain target materials or molecules. These biological applications include, but are not limited to, immunological or other biological assays, biochemical or enzymatic reactions, affinity based protein purifications, cell separations related to cell diagnostics and cell therapeutics, and DNA/RNA isolation in various molecular biology applications.

BACKGROUND OF THE INVENTION

The purification of uncleared, crude materials in a simple, cost effective manner is a

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great challenge to industry. The usefulness of metallic particles, in particular magnetic particles, which have a biological affinity for a substance desired to be removed in such purification processes is well documented. [P. J. Halling and P. Dunning, *Enzyme Microb. Technol.* 2:2-10 (1980); and B. L. Hirschbein et al., *Chemtech*, March 1982: pp. 172-179]. Magnetic particles with covalently bound ligands having an affinity for the substance desired to be separated from a solution are introduced into a mixture containing that substance. After reactions occur between the ligands on the magnetic particle surfaces and the substance, the particles, with the substance bound thereto, are magnetically separated from the mixture. The magnetic particle, with the substance bound thereto, is separated from the solution by applying a magnetic field to the solution. After the magnetic particles, with the substance bound thereto, are removed from the solution, the particle-bound substance is recovered from the magnetic particles by desorbing media. Finally the magnetic particles can be regenerated for reuse, a further economic advantage of the magnetic bioseparation process.

However, the practical development and commercialization of metallic particles has been hindered by several critical properties of the metallic particles. The unprotected surface of metallic particles is often exposed to extreme environments (for example, strong acid, high salt, or high temperature environments) when used in industrial processes. The metallic components of the prior art particles are unstable in these extreme environments. The prior art is replete with attempts to protect the metallic particles from these environments. One method of protecting metallic particles involves coating the particles in glass. Glass coating of particles is widely discussed in the prior art. However, all the processes and methods described in the prior art can not be used to coat particles in the intermediate size range according to the invention.

Particles of an intermediate size range size, as disclosed herein, have a strong particle attraction and/or interaction. These strong interactions make coating intermediate-sized particles difficult. In particular, when magnetic, intermediate-sized particles are attempted to be coated, magnetic and/or dipole-dipole interactions between the particles tend to cause particle agglomeration. Non-magnetic particles of the size range of the present invention will interact as a result of the particles' Brownian motion, which keeps particles in the size range of the invention in constant motion in a colloidal suspension. This constant motion results in frequent inter-particle collisions. These inter-particle collisions lead to induced dipole-induced dipole

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interactions between the particles. These interactions, in turn, cause particle agglomeration even if the particles are non-magnetic. Particle agglomeration is undesired. This problem becomes particularly acute when these particles are coated with an agent that can form cross-links, as this agent will serve to covalently couple particles that interact with one another during the coating procedure. As will be further demonstrated herein, a coating agent that forms cross-links is critical to generating the sealed particles of the invention.

The application of magnetic particles, specifically magnetic particles having biological ligands attached thereto, in bioseparation processes has been actively investigated since 1985. [C. H. Setchell, *J. Chem. Technol. Biotechnol.*, 1985, 35B: pp. 175-182; and C. D. Platsoucas, 1987, *Biomedical Applications of Polymer Particles with Emphasis on Cell Separation (Future Direction in Polymer Colloids)*, M.S. El-Asser and R. M. Fitch (eds.), Marutinus, Nijhoff, Dordrecht, p. 321]. Biomagnetic particles can generally be divided into three classes based on the particle size-- large particles, small particles, and intermediate size particles. Large and small particles have inherent characteristics which make them less preferred than intermediate-sized particles for use in industrial and biological processes.

Large magnetic particles are those particles having a mean diameter greater than about 10 microns (μ). Such particles have the advantage of being able to respond to weak magnetic fields and magnetic field gradients. However, such particles tend to settle rapidly from solution because of their size. Moreover, their large size results in a limited particle surface area per unit mass, which limits the amount of substance which can be bound to each particle. Most importantly, large magnetic particles having a core crystal size of greater than 0.05μ are ferromagnetic, meaning they are permanently magnetized after exposure to an external magnetic field. As a result, large particles aggregate because of the intrinsic magnetic attractions between the permanently magnetized particles. Thus, the applicability of large particles in many biological applications is very limited.

Although Hersh and Yaverbaum in U.S. Pat. No. 3,933,997 report composite particles containing iron oxide (Fe_3O_4) and having a particle diameter of between 1.5 and 10μ, the magnetic particles disclosed therein have an actual size well excess of 10μ in solution, based on the reported binding capacity of 12 mg protein per gram of the particles, and the particle settling rate of 5 minutes. [L. S. Hereto and S. Yaverbaum, *Clin. Chem. Acta*, 63:69-72 (1975); Whitehead et al., U.S. Pat No. 4,695,393, column 3, lines 4 - 26]. Further the

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particles disclosed in U.S. Pat. No. 3,933,997 are silanized according to the methods disclosed in U.S. Pat. No. 3,652,761 to Weetall. [Hersh, U.S. Pat. No. 3,933,997 at column 3, lines 14 - 34]. The methods disclosed in Weetall do not provide a metallic particle that is completely coated by a silica layer. The particles in Weetall and Hersh are in a *porous* silica carrier. Porous coating materials would allow the metallic material enclosed in the porous silanized layer to interact with, or leech into, the hazardous chemical environment. [Weetall at column 4, lines 1 - 18].

There are at least two readily identifiable disadvantages to porous coating of magnetic particles. A porous coat will contain cavities, chambers, tunnels and the like which can non-covalently trap unwanted materials during the separation process. Additionally, both the core material and the coating material are exposed to the sample in a porous coated particle, permitting undesirable interaction between the magnetic core and molecules dissolved in the surrounding medium. Both of these affects lead to increased non-specific binding when compared to the completely coated particles of the invention.

Small magnetic particles, with a magnetic core of a mean diameter less than about 0.03μ, can form a stable colloidal solution and do not spontaneously settle out from solution. As the small magnetic particles have a magnetic core crystal size of less than 0.03μ, they are in general superparamagnetic. Unlike large ferromagnetic particles, superparamagnetic particles only exhibit a magnetic force in the presence of a magnetic field; as a result, superparamagnetic particles do not become permanently magnetized and do not agglomerate absent an external magnetic field.

However, when the small particles are used in magnetic separation systems, the magnetic field strength and magnetic field gradient required to separate such small magnetic particles from solutions, sometimes very viscous solutions, are so large that very large, powerful magnets are required. For example, to separate particles with a diameter of less than 0.03μ, a magnetic field gradient of greater than 20 kGauss/cm is required, greatly increasing the complexity of the required magnetic apparatus. In contrast, particles in the intermediate size range can be separated using relatively weak magnetic field gradients, in the 5 - 20 kGauss/cm range.

Other methods have been tried in the prior art to overcome the weak magnetic characteristics of the small magnetic particles. As one alternative to heavy and bulky magnets,

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thin wires and/or small steel beads are placed inside the reaction containers to generate the required high magnetic gradient internally. However, this approach is inconvenient and often decreases the effectiveness of separation systems. Particles often become entrapped at the inlets of the wire columns, blocking the solution flow through the apparatus.

As small magnetic particles have found some utility, despite their weak magnetic strength, various coating materials have been disclosed for use with small sized particles. However, no material has been utilized which completely protects the magnetic core of a small magnetic particle. Rembaum discloses particles having a diameter between 0.02 and 100 μ. [U.S. Pat. No. 4,267,234]. The particles disclosed by Rembaum are coated with polyglutaraldehyde. Polyglutaraldehyde is a hydrophilic polymer that is readily penetrable by aqueous solution. Thus, components dissolved in aqueous solution will have ready access to the core of these particles. Molday discloses colloidal particles coated with a polysaccharide. [U.S. Pat. No. 4,452,773]. Once again, the coating described in Molday is hydrophilic. These coating materials do not provide sufficient protection to the particles contained therein when placed in hazardous environments.

Intermediate-sized particles are defined as those particles having a particle size between the large particles and small particles described above (between about 0.03 and about 2.0μ in diameter). Although these intermediate-sized particles exhibit preferable magnetic and settling characteristics, the prior art is silent on such particles being made to withstand the extreme environments encountered in many industrial and biological processes.

Whitehead et al. disclose silanized magnetic metal oxide particles of intermediate size which subsequently can be covalently linked to bioactive molecules. [U.S. Pat. No. 4,554,088]. However, the silanized coating on the magnetic particles disclosed in Whitehead is porous, and would not offer proper protection to the metallic core. [U.S. Pat. No. 4,554,088 at column 19, lines 63 - 65]. Czerlinski teaches suspension polymerization of ferromagnetic particles of an average size of no greater than 1.0 μ in diameter. [U.S. Pat. No. 4,454,234]. However, despite their intermediate size, the magnetic particles of Czerlinski will normally agglomerate; Czerlinski teaches making them re-suspendable by heating the particles to a temperature above the Curie temperature of magnetic materials of which the particles are produced, causing the particles to lose their magnetic characteristics. The particles are then coated with an organic (i.e., acrylic) coating material. Margel et al. describe the preparation

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of an intermediate size magnetic and fluorescent particles having a coating of polyacrolein. [U.S. Pat. No. 4,783,336]. These particles have a surface coating layer providing a chemically active substrate for the subsequent coupling of biological materials, such as proteins and enzymes.

Other intermediate-sized magnetic particles have a surface coating of a random coil or globular polymer such as a protein, which is usually absorbed on the particle surface. Owen et al. teach the preparation of polymer coated, colloidal superparamagnetic particles by base co-precipitation of transitional metal oxides in the presence of bioactive polymers such as proteins. [U.S. Pat. No. 4,795,698]. From electron microscopy, the particles are found to be micro-coagulates of one or more magnetic metal oxide crystallites, in a size range of 5-10 nm, which are embedded within a polymer agglomerate. The polymer agglomerate has an overall diameter of about 50 nm. Although these particles exhibit colloidal behavior and have excellent reaction kinetics because of their small particle size, the biological and chemical stability of the particles is less than desirable because the coating materials (for example, bovine serum albumin (BSA)) are loosely adsorbed on the particle surface and may slowly leach out from the particle surface.

The organic coating materials outlined above have several marked disadvantages to the inorganic surface protection layer of the invention. An organic coat can be readily decomposed by oxidizing chemicals and by extreme pH, conditions which do not effect the inorganic coat of the present invention. After the organic coatings of the prior art are dissolved, the metallic particles once enclosed therein are subject to attack by the external environments.

Additionally, the organic coatings of the prior art utilize surface charges to make coating the particles easier. However, these charges on the coated particle surfaces make the particles, once coated, less efficient to use. The coating procedure described in Czerlinski produces a latex particle wherein particle aggregation is inhibited by "charged groups provided by the coating material at the periphery of the particles." [U.S. Pat. No. 4,454,234 at column 2, lines 59 - 61].

These charged groups will convey ion exchange properties to the particle. It is well known in the art of separation sciences that ion exchange properties are undesirable characteristics of an affinity separation particle, as ion exchange greatly increases non-specific binding. In contrast, the procedure described herein produces a base particle with near neutral surface characteristics, when said particles are suspended in neutral aqueous solution.

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Additionally, the present invention allows one to modify the surface characteristics at will. This feature again differentiates particles of the present invention from those coated with prior art organic coatings. While the prior art references do allow for incorporation of reactive functionalities on the surface of the coat, they do not permit production of a particle without an overall negative surface charge.

The intermediate-sized particles of the prior art all suffer from an additional infirmity, their size and shape distributions are not well-controlled. The polydisperse (non-uniform) sized particles of the prior art do not have uniform flow and settling properties when compared to the monodisperse particles of the invention. Additionally, polydisperse particles have a marked increase in non-specific binding as compared to monodisperse particles. Polydisperse particles generate non-uniform cavities on their surfaces when these particles agglomerate during the separation step. This disadvantages of using polydisperse particles in chromatography, for example, is well recognized. Numerous citations in the scientific and patent literature promote the use of monodisperse particles in high performance separation processes, e.g. HPLC. The reaction kinetics of the separation processes require highly uniform particle size and shape.

Well accepted separation theory holds that both entrapment of non-bound compounds, and non-specific binding is minimized when particles of monodisperse size are used in separation processes. Many references disclose the preparation of monodispersed weakly magnetic particles (such as Nickel, Hematite, and Cobalt monodispersed particles) by precipitation from aqueous metal salt solutions. [T. Sugimoto and E. Matijevic, *J. Coll. Interface Sci.* 74: 227-243 (1980); A. E. Regazzoni and E. Matijevic, *Corrosion*, 38: 212-218 (1982); A. E. Regazzoni and E. Matijevic, *Colloid Surf.*, 6: 189-201 (1983); E. Matijevic, *J. Coll. Interface Sci.*, 117: 593-595 (1987); X. J. Fan and E. Matijevic, Patent Application WO 88/05337]. An alternative approach involves the transformation of a monodispersed non-magnetic powder. For example, ellipsoidal hematite (α-Fe_2O_3) particles or dried hematite powder can be converted either to pure metallic iron or maghemite (γ-Fe_2O_3) without changing the particle shape. [M. Ozaki, S. Kratohvil, and E. Matijevic, *J. Colloidal Interface Sci.*, 102 (1984): 146-151]. The procedure for producing the monodispersed particles described in Matijevic involve a complex series of steps and extended reaction times. In contrast, the procedure described herein for producing monodisperse magnetic particles is straightforward and can be completed within minutes. Moreover, the magnetic particles produced according

to Matijevic are of uniform size and shape, the particles are uncoated and, therefore, irreversibly aggregate in solution because of inter-actions between the bare metal oxide surfaces of the particles. Thus, the Matijevic procedure does not produce an intermediate-sized particle which can be maintained in colloidal suspension, but instead produces an aggregate. These aggregates are highly polydispersed in size and shape.

Ughelstad made progress in obtaining monodispersed, and uniformly shaped, spherical magnetic particles. [U. Ughelstad, T. Ellingsen, and A. Helgee, International Patent Application WO83/03920; J. Ughelstad et al., *Prog. Polymer Sci.*, 17: 87-161 (1992); J. Ughelstad et al., *Adv. Colloid Interface Sci.*, 13: 1201-1234 (1980); J. Ughelstad et al., *Sci. Technol. Polymer Colloids*, NATO ASI Series 17. Boston, Nijhoff, pp 51-99 (1980); and J. Ughelstad et al., *Blood Purif.*, 11: 349-369 (1993)]. The magnetic polymer particles disclosed by Ughelstad, sold commercially as Dynabeads, have a uniform diameter. However, they do not have uniform magnetic properties. The Dynabead production process involves the precipitation of magnetic metal inside a porous latex particle, which is then sealed with a latex barrier coat. Even those latex particles that possess a uniform diameter have internal cavities of differing size and shape. Hence, the resulting precipitated magnetic component will be widely variable from particle to particle. Additionally, Dynabeads are based on an organic backbone coat and thus suffer from all the disadvantages of organic barriers previously discussed.

European Patent Application No. 90118468.9 to Okada discloses magnetized polymer particles of 0.2μ to 3.0 μ in diameter comprising an organic polymer core and an iron oxide (ferrite) coating layer. No outer protective coating barrier layer is taught in this reference. The particles are of well-controlled size and shape. However, the unprotected ferrite surface of the particles is chemically and physically unstable in extreme environments (strong acid, high salt, or high temperature).

U. S. Pat. No. 4,358,388 to Daniel et. al. discloses preparing polymer-coated metal oxide spherical particles by emulsion polymerization of vinyl aromatic monomers in the presence of magnetic seed particles. The resulting magnetic polymer spheres exhibit a wide particle size distribution and magnetic content. Additionally, the method disclosed by Daniel requires the use of organic based co-precipitants and coats.

U.S. Pat. No. 5,062,991 to Simian teaches a method for making colloidal particles of

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ferrites containing manganese(II), zinc(II), barium(II), iron(II), or nickel(II) in a gelatin solution which acts as a support vehicle for nucleation and growth of the colloidal metal oxide particles. These magnetic particles have spherical shapes, and reasonably uniform sub-micron particle sizes. However, the bare metal oxides are not well protected by the gelatin, resulting in limited chemical stability of the particles in saline or strongly acidic conditions.

OBJECTS OF THE INVENTION

It is an object of the invention to provide metallic particles of intermediate size which are stable in aggressive environments, such as highly acidic, basic or saline environments.

It is a further object of this invention to provide magnetic particles of an intermediate size which are capable of being used in separation processes. The particles should be of uniform size and shape. Preferably, the particles should exhibit superparamagnetic behavior.

It is a further object of the invention to provide magnetic particles which have a high affinity for biological ligands, to which ligands substances are bound in a separation process, thereby allowing removal of the substance in the separation process.

It is a further object of the invention to provide a method of producing such magnetic particles.

It is a still further object of the invention to provide a method of using such particles in separation processes.

Other objects and advantages of the invention will become apparent to those skilled in the art from the accompanying description of the invention.

SUMMARY OF THE INVENTION

The invention provides well controlled metallic particles comprising a metallic core surrounded by a sealed inorganic surface protection layer to which a wide variety of molecules can be coupled. The metallic particles of the invention are of uniform, intermediate size. When the metallic particles of the invention comprise magnetic metal or magnetic metal oxide the particles exhibit superparamagnetic characteristics. The particles of the invention offer significant advantages over prior art particles with respect to chemical, biological and colloidal stabilities. In order to produce the sealed, inorganic surface protection layer of the particles of the present invention, while avoiding particle agglomeration, it is necessary to utilize barrier-forming agents that will form multiple cross-links within the barrier. This requirement for cross-linking in the barrier material is a great advance over the prior art technology. It has been

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heretofore unknown to apply cross-linking materials to particles in the intermediate size range of the invention as the interacting particles in this size range will encourage particle-to-particle cross-linking upon application of the cross-linking materials, which will lead to massive, covalently linked aggregates.

This cross-linked surface protective layer completely coats the metallic core of the particles according to the invention. Preferably the surface protective layer is comprised of silica. The preferred silica surface protective layer prevents the metallic core of the particles of the invention from leaching into or reacting with a solution which bathes the particles.

According to the invention, the metallic material of the particles is covered in a homogeneous layer of rigid inorganic material, preferably silica (SiO_2) or silica containing materials, e.g., glass. The metallic materials are sealed by the rigid inorganic materials. Sealing enhances the dispensability and resistance to coagulation of the particles, and provides a surface protection layer that prevents both reaction of the metallic material core with the surrounding medium, and leeching of the metallic material core to the surrounding medium. The sealing properties of the glass layer are of great importance as the surrounding media in bioseparation processes can provide extremely hazardous environments for the metallic material core.

The formation of a glass surface protective layer around the metallic core may be carried out by dehydration of TEOS in the presence of the metallic material cores, followed by heating or dehydration to further remove water and to ensure the formation of Si=O bonds in the coating. Two factors are critical in preparing coatings according to the present invention -- the concentration of the coating materials and the rate of coat formation. The relative concentrations of magnetic particles and coating materials must be carefully controlled to maximize the rate of coat formation compared to the rate of particle interaction. This control of relative rates also necessitates careful control of other reaction components and conditions, for example, water and catalyst content and reaction temperature. Additionally, particles prepared according to the present invention are vigorously agitated during the coating step. Preferably, this agitation is accomplished by means of sonication. However, mechanical homogenization means can be used in the practice of the current invention.

The outer surface of the protective layer of the metallic particles can be further modified to form functional groups, including, but not limited to, amine groups ($-NH_2$) and carboxyl

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groups (-COOH), on the outer surface. These functional groups enable a wide range of biologically active materials, or "ligands", (including, but not limited to, proteins, polymers, copolymers, synthetic polypeptides, enzymes, nucleotides and combinations thereof), to be coupled covalently to the metallic particle's surface, using conventional coupling chemistries. Since the ligands are linked to the functionally-activated particle by covalent bonding, the biological stability of the particles is ensured. As a result, the biologically active metallic particles are useful in immunoassays or other binding assays for the measurement of analytes in test media, cell separations, protein purifications, affinity based chromatography, and the like.

The metallic portions of the particles according to the invention comprise cores comprising magnetic and/or non-magnetic metallic materials. When the particles are magnetic, the metallic cores can be pure metals which exhibit magnetic behavior and/or metal oxides. A magnetic metallic core is produced in a kinetically controlled oxidative hydrolysis of a metal salt solution, such as $FeSO_4$, in an alkaline media. An alternative method utilizes a base precipitation method wherein air is rigorously excluded, thereby avoiding partial oxidation and poor crystallization of the resulting magnetic cores.

Metallic particles, in particular, magnetic metallic particles, with streptavidin or avidin bound to the modified glass surface thereof are particularly useful in a number of biological applications. Since biotin binds readily with the streptavidin/avidin on the particle surface, such streptavidin/avidin coated particles can be used to effectively capture, and thereafter separate, any biological materials with biotin-tags or those linked by biotinylated vectors such as biotinylated monoclonal antibodies (CD4, CD8, CD45, CD34, et al.) and biotinylated oligonucleotides (dT_{25}). According to one embodiment of the invention, biotin is covalently attached to amine ($-NH_2$) groups on particle surface using common biotinylation technique. Streptavidin or avidin is then coupled to the biotin-tagged particles. The bond formation between biotin and streptavidin/avidin is very rapid and, once formed, is unaffected by extreme pH, organic solvents, and other denaturing agents, because the streptavidin/avidin-biotin reaction is one of the strongest known non-covalent biological recognition reaction ($K_a = 10^{-15}$). Therefore, streptavidin/avidin coated metallic particles of the present invention can provide a powerful and versatile tool for use in, among other processes, immunoassays, biological cell separations, receptor studies, DNA/RNA isolation, and protein purification.

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Magnetic metallic particles according to the invention are particularly useful for antibody purifications, and for DNA/RNA isolation. Because the inorganic surface protective layer completely surrounds the magnetic core of the particles according to the invention, the magnetic particles are stable in both antibody purifications and DNA/RNA isolation systems which may present rigorous conditions to the particles, for example, high temperatures during PCR amplification or high salt concentrations during antibody or mRNA binding steps. Being sparedfrom contact with these harsh environments, the metallic particles of the invention may be reused. In the case of mRNA isolation, after removing the particles from the surrounding medium and elution of the mRNA from the particles, the magnetic particles are washed several times and then reconditioned and transferred to an RNase-free storage buffer for reuse.

According to one aspect of this invention, metallic particles having functional groups, such as amine (-NH2) groups or carboxyl (-COOH) groups, on the particles' outer surfaces (via modification of the inorganic surface protective layer), have transitional metal ions (Ni, Co, Co, Zn, Fe, et al.) bound to the functional groups. Such metallic particles are particularly useful when nickel ions are bound to the functional groups on the outer surface of the particle. Nickel coated particles are particularly useful and versatile for isolating recombinant, 6xHis-tagged proteins and peptides. This protein purification technique is based on the high affinity of histidine residues of recombinant proteins and peptides for nickel ions. Nickel coated metallic particles are used in separation methods to provide true one-step purification of almost any protein under native or denaturing conditions. Other applications using Nickel coated metallic particles include efficient detection, immobilization, assay, and high-level bacterial expression of 6xHis-tagged proteins.

A further novel aspect of the present invention is the production of particles within the indicated size range that have a monodisperse size distribution. The preparation of the magnetic core particles according to the invention utilizes seeded growth involving heterogeneous nucleations, in which inorganic nucleation seeds with a well-defined shape and a relatively narrow size distribution are used as a starting nucleation substrate to control the shape and size distribution of the resulting magnetic cores. A wide range of materials, such as metal hydrous oxides (hematite, goethite, lepidocrocite, feroxyhyte, akaganeite, et al.), well controlled pure metal particles (nickel, copper, cobalt, zinc, iron, et al.), and pure silica (SiO_2) or silica-containing particles, may be used as nucleation seeds. The embedded inorganic nucleation

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seeds enable one to custom design and synthesize magnetic particles with selected physical characteristics such as size, density and magnetic susceptibility.

Preferably, the nucleation seeds are metal oxide particles or silica particles. The desired size distribution and magnetic properties of the magnetic core are obtained by controlling the concentration and size of the nucleation seed. Small uniform metal hydrous oxide seeds are prepared by employing a reaction system in which nucleation and crystal growth during the synthesis are separated. This separation of nucleation and crystal growth is achieved by arranging the reaction conditions of seed growth to allow a slow release of the ionic species of the seed until the critical supersaturation level required for nucleation is exceeded. Preferably, the seeds are small, uniform silica particles (SiO_2) of spherical shape that are prepared using tetraethylorthosilicate (TEOS) in an water-alcohol mixture with an ammonia catalyst.

Particles prepared according to the invention exhibit minimal settling during separation processes. The biomagnetic particles (magnetic particles with biological ligands attached) are preferably between 0.08μ to 1.2μ in diameter, which enables them to maintain dispersed in media for sufficient time periods to allow them to perform their intended biological separation functions. Because of their relatively small particle size and their resulting relatively large surface area (as high as 125 to 185 m^2/gm of particles), the particles of the invention have a high capacity for the biological ligands. Further, the colloidally stable magnetic particles provide fast reaction kinetics, and allow more magnetic particles to be bound to the target species (cells, bacteria, antibodies, et al.) as compared to larger particles such as Dynabeads M-450 (4.5μ in diameter). The magnetic particles according to the invention are superparamagnetic, therefore they do not exhibit magnetically induced aggregation, can be re-suspended and reused, and are easily removed from the separation solution, with less powerful magnets than those required to remove larger-sized particles. The magnetic particles in the intermediate size range of the invention can be retrieved from separation apparatus with a magnetic gradient field generated by a single permanent magnet.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Although the present invention extends to metallic particles of both a magnetic and non-magnetic nature, the preferred embodiment of the invention comprises a magnetic particle to be utilized in magnetic affinity, bio-separation processes. In the preferred embodiment, the metallic material of the particle comprises a metal oxide. Preferably the magnetic particle is

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of uniform size and shape.

Methods of obtaining monodispersed metal oxide particles, which result in well defined colloidal metal oxide particles are known in the art. [M. A. Belsa and E. Matijevic, *Adv. Colloid Interface Sci.*, 29 (1989): 173-221]. A large number of uniform nonmagnetic, or weakly magnetic, powders of different chemical compositions have been prepared in a variety of particle shapes (such as SiO_2, TiO_2, Al_2O_3, α-Fe_2O_3, $CuCl_2$, SnO_2, $NaSO_4$, $Ce(SO_4)_2$, $Fe(NO_3)_3$, $FeCl_3$, et al.). However, problems still exist in producing monodispersed, strongly magnetic particles, for example, monodispersed particles of maghemite and magnetite. In accordance with the present invention, there is provided an improved method of producing magnetic particles with a narrow size distribution, as well as desirable particle density and magnetic susceptibility.

Formation of intermediate-sized particles of uniform size in aqueous systems involves two phases -- nucleation and crystal growth. Particles with wide deviation in their particle sizes are usually an indication that the nucleation and crystal growth steps took place simultaneously over the bulk of the reaction involved in the particles' production. To obtain particles with a narrow size distribution, nucleation and crystal growth must be separated.

There are two types of nucleation -- homogeneous nucleation and heterogeneous nucleation. Both types of nucleation may exist in a metal oxide system during particle formation, depending upon the parameters of the reaction. Homogeneous nucleation occurs spontaneously in bulk solutions at supersaturation, when the ion activity product of a compound exceeds its solubility product. The essential requirement for precipitation to take place is the formation of stable, embryonic clusters, or nuclei, in solution. The stability of an embryo and the nucleation rate, J_N, are both related to the free energy of nucleation, E_N, which is the sum of the free energy, $\Delta E_{surface}$, for the creation of a new interface, and the energy, ΔE_{bulk}, released by the formation of bonds in the bulk structure. Only when the embryo exceeds a certain critical size, does ΔE_{bulk}, predominate. This size defines the critical nucleus, i.e. the smallest embryo that can continue to grow by a decrease in total free energy E_N. The rate of nucleation is usually very slow until critical supersaturation is achieved, after which J_N rises rapidly.

Heterogeneous nucleation, on the other hand, occurs when the presence of a solid phase substrate in the reaction system reduces the total free energy of nucleation, thus increasing the nucleation rate. The solid phase substrate can be fine seeds of a foreign material or crystal

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seeds of the primary material which is crystallizing. If the interfacial free energy between the crystal face of the primary material and the substrate is lower than the interfacial free energy between the crystal face of the primary material and the reaction solution, nucleation and crystal growth occur at the surface of the solid phase substrate. Heterogeneous nucleation is energetically favorable when using a solid phase a substrate with a structure type and an inter-atomic distance that match the structure type and inter-atomic distance of the primary material being crystallized. According to this invention, seed crystals with sufficiently uniform size are added to the reaction system in which solute release is extremely slow and localized crystal growth occurs around the seed crystals, bypassing the nucleation step. The use of seed crystals to produce uniform particles is critical to the invention. Although the use of seed crystals to control nucleation growth has been discussed in the prior art, seed crystals have not been utilized to produce metallic and/or magnetic intermediate-sized particles of monodispersed diameter.

Different from nucleation, crystal growth requires a lower degree of supersaturation, and is mainly controlled by transport processes, rate of surface chemical reaction, and solution conditions (pH value, ionic strength, temperature, aging time, the nature of the anions, et al.). In general, crystal growth involves five steps: 1) diffusion of ions or molecules to the surface of the crystal, 2) partial or complete dehydration of ions at the surface, 3) adsorption at the nucleus' surface, 4) two-dimensional diffusion over the surface to an energetically more favorable position - a kink or screw dislocation, and 5) one dimensional diffusion along the kink to a site where the ion is incorporated into the crystal.

Producing uniform-sized particles in a well controlled and reproducible manner has not been accomplished, prior to the invention. According to the invention, inorganic seed particles are utilized as the substrate to separate nucleation and crystal growth and to diminish secondary nucleation. The seed particles serve as a substrate for heterogeneous nucleation of the magnetic cores of the particles of the invention. Metal hydrous oxide particles and silica particles may be used as the preferred inorganic seeds, and magnetic transition metal oxides are the preferred component of the magnetic core. To produce particles according to the invention, the rate of solute introduction is slowed down so that the solute is removed from solution entirely by adsorption onto existing nuclei. Therefore, the concentration never reaches the level required for further nucleation to take place.

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Preparation of Inorganic Seed Particles

Inorganic seed particles having utility in the present invention can be prepared by a number of established procedures, for example the method disclosed in PCT application number WO88/05337 to Fan and Matijevic. The metal oxide seed preparation methods disclosed herein are taken from T. Sugimoto and E. Matijevic, *J. Coll. Interface Sci.* 74: 227-243 (1980). The metal hydrous (hydroxide or oxide) seed particles of this invention are prepared primarily by forced hydrolysis of metal salts in an acidic aqueous solution and aging the metal salt solution at elevated temperatures. Spherical colloidal hematite (α-Fe_2O_3) particles of narrow size distribution are utilized as seed particles in one embodiment of the invention. Fine hematite particles are an ideal seed substrate material for controlling the crystal growth of magnetic metal oxide cores, because of the high degree of similarity between the structure type and inter-atomic distances of hematite and the materials utilized to form the magnetic core. This matching of the structure and interatomic distances of the inorganic seed and the metallic core material is essential to producing the monodisperse particles of the invention, not any chemical similarity between the specific metal oxide and hematite seed.

Forced hydrolysis of an acid Fe^{3+} solution involves hydrolyzing $Fe(NO_3)_3$, $Fe(ClO_4)_3$, $Fe(SO_4)_3$ or $FeCl_3$ solution at about 100 °C under strongly acidic conditions (pH 1-2), and aging the solution for varying periods of time (from 30 minutes to several weeks). With the exception of $Fe(SO_4)_3$, the iron (III) salts were dissolved at room temperature to minimize hydrolysis. $Fe(SO_4)_3$ was heated to 40 °C with rapid stirring to prepare an iron salt solution of the required concentration. It is preferred to prepare concentrated stock solutions which can be kept for long periods of time. All solutions are filtered through 0.45μ filters before use. All sols described here are made by heating solutions of ferric (Fe^{3+}) salts for different periods of time. In order to produce narrowly distributed, spherical hematite seed particles, the concentration of ferric ions and the respective anions, the pH, and the temperature of reaction system must be carefully controlled. Stock solutions of ferric salts are mixed with predetermined amounts of the corresponding acids prior to adding water, to avoid hydrolysis upon dilution. The pH range varies between 0.5 to 2.5, measured at room temperature before and after aging the solution. Aging is carried out at elevated temperatures in a constant temperature oven. The aging temperature range is from 50 to 100°C. After aging, the aged samples are cooled to room temperature. Then the precipitate of hematite particles is washed with water using conventional

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techniques of centrifugation or filtration, and redispersed until a neutral pH is reached. The final hematite particles can be stored in water.

A variety of commonly used analytical techniques may be employed to characterize the composition, size, and the shape of the particles. The most important techniques include X-ray powder diffraction, electron microscopy, and dynamic laser scattering. X-ray powder diffraction is the most useful analytical tool for identifying the purity of the final product. In addition, it also provides useful information on crystal size and disorder, structural parameters (unit cell edge length), degree of isomorphous substitution and surface area. Electron microscopy techniques provide direct measurement of particle dimension and crystal morphology. Mean diameter, size distribution, and diffusion coefficient of the particles can be conveniently measured by particle sizers using dynamic laser scattering method. The particle size and size distribution described in this invention are primarily obtained by dynamic laser scattering method.

By using the procedure described above and by properly selecting the reaction parameters, a number of metal hydrous oxide seeds having a narrow size distribution can be produced. Other metal salts, such as those containing Mn, Mg, Co, Ni, Zn, Cu, Al, Ti, Mb, Mo, Pd, Ag, Cd, Gd, Tb, Dy, Er, Tm, Hg, V, and combinations of two or more of these metals, may be substituted for iron (III) salts to produce a variety of well defined metal hydrous oxide particles with different shapes or crystalline structures depending on the type of anions present in the aged solution. For example, well defined spherical aluminum hydrous oxide particles of 1.0μ in diameter are obtained by aging $KAl(SO_4)_2$ at 100 °C for 24 hours. Thus, using different metal salts yields a spectrum of metal hydrous oxide particles with not only different chemical compositions, sizes and shapes, but different densities and magnetic susceptibilities, as well.

A second type of seed used in this invention comprises fine spherical silica particles of uniform size. Using well defined silica seed particles as an alternative to fine metal hydrous oxide seeds provides important conveniences. Besides simplicity and ease of manufacturing, the high dispersibility of silica particles in aqueous solutions, and the high density of hydroxyl groups (OH) on the silica seed particle surface, make silica seeds very attractive. Many methods for the preparation of monodispersed silica particles of different diameters are established in the art. [E. Wagner and H. Brunner, *Amer. Chem.*, 72 (1960): 744, W. Stober, A. Fink, and E.

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Bohn, *J. Coll. Interface Sci.*, 26 (1968): 62-69]. According to one embodiment of this invention, fine silica seed particles are prepared by the method of Wagner et al. by hydrolysis of alkyl silicates and subsequent condensation of silicic acid in alcoholic solutions in the presence of ammonia as a morphological catalyst, which prompts the formation of spherical particles.

Analytical grade methanol, ethanol, *n*-propanol and *n*-butanol are used as solvents for the reaction. Tetraesters of silicic acid (tetraalkylsilicates) are used or can be prepared by reacting silicon tetrachloride with alcohol (*n*-propyl, *n*-butyl, *n*-pentyl). Saturated alcoholic ammonia solution is often used as one of the reaction solvents, and can be prepared by passing ultra pure ammonia (anhydrous 99.99%) through a dry column filled with sodium hydroxide pellets and then bubbling the eluent through the alcohol at 0°C . However, highly concentrated ammonium hydroxide solution is preferred. The reagents are mixed according to the desired concentrations of water and ammonia. Before adding the tetraester of silicic acid, the concentration of ammonia and water are carefully measured. Then, the tetraester of silicic acid is added and mixed with the premixed reagents in a reaction vessel. The concentration of each reactant, the temperature, and pH are carefully controlled such that the system is supersaturated and homogeneous nucleation occurs, permitting the formation of well defined silica particles of uniform size. Adequate mixing during seed particle synthesis is essential, and can be achieved by utilizing a number of common laboratory techniques. Total reaction times may vary between 10 minutes and several hours depending on the desired seed particle size (0.05μ to 2.0μ). Finally, the precipitate of silica seed particles are washed with water by conventional techniques such as centrifugation and filtration, and redispersed repeatedly until a neutral pH is reached. The final silica seed particles can be stored in water as a colloidal solution or can be dried up to form powders.

The seed particle preparation methods disclosed above are among a multitude of preparation methodologies which can be used to prepare seed particles having utility in the present invention and the aforementioned procedures are not intended to limit the present invention in any way. Any seed particle containing monodisperse size distribution within the range of seed particle sizes useful in the present invention, 0.003 to 0.8 μ, can be substituted for the particles produced according to the above methods without departing from the scope and intent of the present invention.

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Since metal hydrous oxide particles or fine silica particles can be used as seeds to host and control crystal growth of the magnetic cores, the wide range of different metal hydrous oxide particles and silica particles provides the ability to custom design and synthesize magnetic particles with specific physical characteristics (including size, density and magnetic susceptibility). Such design specificity is important, depending on the materials to be separated in the magnetic affinity separation process. Often times the separated materials are cells that are often crushed and/or damaged during the separation process by particles that are too large or too magnetic for the material to be separated. Hence, by properly selecting the mass ratio between the magnetic component and the non-magnetic component (when silica is the seed material), or less magnetic component (like hematite), of the embedded seeds, it is conceivable that for a given particle size, the total magnetic momentum of a composite magnetic particle can be adjusted to optimum value to ensure gentle magnetic separation processes.

Preparation of Composite Magnetic Core Particles

Composite magnetic core particles according to the present invention are preferably prepared by the precipitation of transition metal oxides in aqueous solutions in the presence of the well defined fine inorganic seeds. This is a seeded crystal growth of the metallic material involving heterogeneous nucleations.

The process for growing the magnetic core involves two steps. A metal salt is hydrolyzed to produce a metal oxide or hydroxide which has limited solubility in aqueous solution. This oxide or hydroxide then precipitates on the seed particle, growing the magnetic core. In general, the metal hydroxylation process is sensitive to many reaction conditions, including pH, temperature, concentration of reactants, nature of anions, storing, and aging time. As a result, it is possible to regulate the rate of hydrolyzed metal ion complex formation in aqueous metal salt solutions by proper selection and control of the reaction conditions. For example, elevated temperatures accelerate hydroxylation greatly and the hydrolyzed metal ion complexes form in solution at acidic conditions (low pH). After nucleation begins (once the solution becomes supersaturated with respect to the constituent species of a given metal hydrous oxide), further aging at the proper temperature enables continuous precipitation of hydrolyzed metal ion complexes for particle growth. Consequently, secondary nucleation is avoided and a uniform uptake of the metal oxides on the seed surfaces yields well defined particles.

According to one embodiment of the present invention, composite magnetite cores are

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prepared through the oxidative hydrolysis of iron (II) salt solutions (typically $FeSO_4$ or $FeCl_2$) in an alkaline media in the presence of fine inorganic crystal seeds. This method allows the production of composite magnetic particles with selected physical characteristics, including shape, size, density, and magnetic susceptibility. The final product depends on synthesis conditions, in particular pH, iron salt concentration, temperature, the nature of non-constituent anions and cations in the reaction system, size and concentration of seed particles, and the method of agitation.

Preferably, the whole reaction is carried out under an inert gas such as N_2. The reaction can be carried out with the pH range 7-14. However, magnetite particles can be obtained only at pH values between 9 and 12. During the oxidation / hydrolysis reaction, as the formation of iron hydrous oxide proceeds, the pH decreases due to the release of protons from the following reaction:

$$(2Fe^{2+} + 3H_2O + 1/2\ {}_2 \rightarrow\ 2FeOOH + 4H^+).$$

If no additional base is added to the system, incomplete oxidation occurs. Therefore, in order to ensure a reasonable crystalline product with a high yield of magnetite, the pH in reaction system must be held constant by continual addition of base to the system. Alternatively, buffers such as $NaOHCO_3$ or imidazole may be used.

In the present invention, one preferred process for the formation of composite magnetite cores is started by adding a solution containing inorganic crystal seeds, for example fine hematite or silica particles of a given concentration, to aqueous solution of an iron (II) salt which has been previously flushed with N_2 gas. The size of the crystal seeds and the relative proportion of ferrous (Fe^{2+}) salts and the seed materials in starting solution are two critical factors in determining the morphology, density, and magnetic property of final composite magnetic cores. The whole reaction system is kept under air free conditions by continual purging of N_2 during the entire reaction. A constant, elevated reaction temperature is preferred and achieved by placing the reaction vessel in a constant temperature water bath. A temperature range of 37 to 95°C is desired, preferably 75 -85°C. Higher temperatures are preferred for kinetic reasons. It is critical to maintain the temperature at a constant level as any variation in reaction temperature reduces the probability of forming monodisperse sized particles.

After reaching the reaction temperature, the mixture of ferrous salt and fine seed particles is added to an oxygen-free solution of KOH and KNO_3 (0.1 to 5M, 0.5 to 1M

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preferred, for each solution). This reaction produces a gelatinous suspension of $Fe(OH)_2$ which is kept under elevated temperature for aging in the presence of ferrous ions for a time period of 30 minutes to one week, during which time black magnetite precipitates. The resulting composite magnetite core particles are then cooled to room temperature and washed several times with distilled water by repeatedly dispersing the precipitate in an ultrasonic bath after separating the precipitate from the solution with a magnetic field.

Variation of the different reaction parameters effects the characteristics of the magnetic cores. The nature of anion in the iron salt plays a significant role in the crystallization process. The resulting magnetic core particles from solutions of different salts of the same metal ion may have different compositions and structures. For example, precipitation of an $Fe(NH_4)_2(SO_4)_2$ solution results in rather large magnetite particles with a heterogeneous size distribution when ferrous ions are in slight excess in basic solutions. Aging ferrous chloride ($FeCl_2$) solution yields very small magnetite precipitate (less than 0.02μ) in the presence of KNO_3. Non magnetic particles are produced if ferrous acetate $Fe(CH_2CO_2)_2$ is used as a core material. When using ferrous sulfate $FeSO_4$ as the core material, the concentration of iron (II) species is a critical parameter in determining the size distribution of resulting magnetite particles. The most uniform dispersion can be obtained at a small excess of ferrous ions, while a large surplus of free ions results in a narrow size distribution. Different aging temperatures may affect the rate of magnetite crystallization and may also yield final particles with different sizes. Furthermore, any agitation of the system during aging step can cause a broadening of size distribution.

Another preferred process of producing composite magnetite cores comprises precipitation of ferrous and ferric salts in the presence of inorganic seeds. This reaction is performed at a pH in the range of 9-10, using strong base such as sodium hydroxide (NaOH) or ammonium hydroxide (NH_4OH). To prevent poor crystallization during the reaction, air must be rigorously excluded. To achieve air exclusion, all reagents, including the staring reaction mixture, are bathed in pure nitrogen gas for at least one hour before use to remove any dissolved oxygen, and the reaction process is carried out under constant N_2 bathing as well.

The preferred ratio of ferrous ion and ferric ion is 2:1. However, other ferrous/ ferric ratios ranging from 1:2 to 10:1 may also be employed to produce satisfactory magnetic cores while keeping a constant total amount of iron. The reaction temperature is preferably between 50 to 90 °C to ensure maximum yield of magnetite. Uniform mixing during precipitation can

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be achieved with vigorous agitation, such as by rapid stirring or ultrasonication.

The precipitant black magnetite particles are repeatedly washed with distilled water until a neutral pH is achieved. Between washing steps the particles are removed from the wash fluid by using a magnetic field. Subsequently, the washed composite magnetite particles can resuspended in distilled water or a low ionic strength buffer medium at neutral pH, for example, sodium phosphate, HEPES, borate or sulfate systems.

Besides divalent and trivalent iron salts, other magnetic components may be used as the core material. For example, any divalent transition metal ion, including but not limited to Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cu^{2+} may be used for the preparation of composite magnetic cores in the processes listed above instead of ferrous ions. A preferred class of magnetic oxides known as ferrite can be used as the core material. Generally, ferrites are represented as ($MO_x \cdot Fe_{3-x}O_3$) in which M is Mg, Co, Cu, In, Fe, V, Gd, Zn. A preferred ferrite is magnetite ($FeO \cdot Fe_3O_3$). According to one embodiment of this invention, other non-transition metal elements may be utilized to form non-ferrite type metal oxide cores using a combination of one or more of the following metal ions: Al^{3+}, Ti^{4+}, V^{3+}, Mb^{2+}, Co^{2+}, Ni^{2+}, Mo^{5+}, Pd^{3+}, Ag^{+}, Cd, Gd^{3+}, Tb^{3+}, Dy^{3+}, Er^{3+}, Tm^{3+}, and Hg^{+}. These metal ion species are also introduced into the aqueous reaction solution in the form of metal salts. Methods similar to the one described above have been widely used in the preparation of magnetic particles. However, the method of this embodiment of the invention differs from prior art processes in at least two critical ways -- the seed crystals and the exclusion of oxygen during the precipitation reaction. Both of these critical factors are required to produce the monodispersed particles of the invention.

Possessing the benefits of seeded crystal growth, composite magnetic cores prepared according to the present invention exhibit many useful properties, and overcome problems associated with size and size uniformity -- namely, gravitational settling, surface area, density, and magnetic characteristics -- of the magnetic particles of the prior art. Particles according to the invention have a narrow size distribution. Within the size range claimed in this invention (0.03μ - 2.0μ), the composite magnetic particles, with or without coating, are colloidally stable in solutions as a result of only the particles' thermal motion because the silica seeds deposited within the magnetic cores reduce the overall particle density. The composite magnetic cores produced according to the preferred embodiment of the present invention are

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superparamagnetic, and do not exhibit any bulk magnetic properties after the withdrawal of a applied magnetic field. Since the ratio of the masses of the magnetic component and the non-magnetic (or less magnetic) component of a composite magnetic core is controlled under the above process, the magnetic susceptibility of the magnetic core can be adjusted to a required specific value to meet the requirements for any intended applications.

Casting of Protection Layer around Composite Magnetic Cores

The composite magnetic (or non-magnetic) cores of the particles according to the present invention are sealed by an inorganic surface protective layer. The sealed protective layer meets several requirements: the coating layer must be rigid and must completely seal the metallic core; the thickness of coating must be a easily controlled; the protective layer must be chemically resilient, especially to strong acid and base, high ionic strength, elevated temperature and the like, and the protective layer must contain certain chemical functionalities, which can be modified for attaching biological materials such as proteins, oligonucleotides, enzymes, et al.. According to the present invention, the preferred inorganic protective layer comprises crystalline or non-crystalline silica (SiO_2) and other silica derivatives. Silica is chosen as the layer substance in this invention because it meets the above requirements. Further according to the present invention, a new and novel approach is invented, by which a compact surface protective layer of silica can be grown directly on the composite metal oxide cores once the system is supersaturated with respect to the coating materials.

The general formula of inorganic silicate coating agents that may be used to form a silica layer on the metal oxide surface of the metallic core is represented by SiX_n, where the X group generally reacts with the metal oxide core material. The bond between X and the Si atom in the coating agent is replaced by a bond between the metal oxide core and the Si atom. X is a hydrolyzable group, typically, alkoxy, acyloxy, amine, or chlorine. Methoxy and ethoxy groups produce methanol and ethanol, respectively, as byproducts during the silica layer forming reactions. In the preferred embodiment of the invention, tetraethylorthosilicate (TEOS) is utilized as a source of silicon dioxide or silica. In the case where tetraethylorthosilicate ($Si(OC_2H_5)_4$) is used as silica coupling agent, X is an ethoxy group (OC_2H_5). The hydrolysis reaction yields polymers of silicic acid which can be further dehydrated to silica. This is the primary reaction pathway in the silica coating step of this invention. Such presumed chemical reaction is

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$$Fe_3O_4 + Si(OC_2H_5)_4 + NH_3 + 2H_2O \rightarrow Fe_3O_4\text{-}SiO2 + 4C_2H_5OH + NH_3.$$

The bond between the Si atom and the metal oxide core is not formed directly during hydrolysis. Intermediate ethoxy derivatives of silicic acid and polysilicates form as the hydrolysis reaction progresses. Following hydrolysis, reactive silano groups (SiOH) are formed which condense with other silano groups to form siloxane linkages. The polysilicates grow in molecular weight and chain length until most or all of the ethoxy groups are removed and a network of -Si-O-Si- remains. TEOS is generally partially hydrolyzed and comprises a cyclic and linear structure of 3-6 Si atoms. Partially hydrolyzed materials of this type often contain sufficient silano groups (SiOH) to displace most of the remaining ethoxy groups during an acid or base catalyzed condensation.

According to the present invention, the preferred silica coating process involves four steps: hydrolysis of the three X groups attached to Si; condensation to oligomers; hydrogen bonding between oligomers and the OH group of the silanol; and during drying or curing, a covalent linkage is formed between the Si atom and the metal oxide core with concomitant loss of water. At the Si-metal oxide interface, there is usually only one bond from each Si of the silica coupling to the metal oxide surface. The remaining silano (SiOH) groups are either bonded to other silicon atoms or in free form. Once the reaction system is supersaturated with respect to the coating material, silica crystals (in reality, a compact non-linear silica network) can be grown directly on the composite metal oxide cores. The protective layer thickness can be controlled by controlling the reaction conditions (including temperature, reaction time, the concentration of coating materials, et al.). At any given concentration for coating reagents, reaction time is the dominant parameter controlling coating thickness. Longer reaction time yields thicker protective layers. The hydrolysis reaction can be acid or base catalyzed. For example, dilute hydrochloric, nitric or acetic acids can be used to facilitate formation of stable silanol condensation products. When more complete condensation is preferred, a wide range of catalysts, including moderate basic ones is employed. The preferred materials used as hardeners or accelerators are aqueous or anhydrous ammonium, ammonium hydroxide, ammonium carbonate, triethanolamine, calcium hydroxide, magnesium oxide, dicyclohexylamine, alcoholic ammonium acetate and tributyrin oxide. To promote rigid glass formation on the core, a strong base such as sodium methoxide, sodium hydroxide or lithium methoxide may be added.

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A preferred procedure for forming silica protective layers follows: To a solution of ammonia (0.0 - 10.0 mol dm^{-3} NH_3) and water (0.5 - 30 mol dm^{-3} H_2O) in alcohol, a predetermined amount of composite metal oxide core particles (5 - 100 mg dm^{-3}) is added to a tightly screw-capped reaction vessel under constant agitation. The vessel is then placed in a constant temperature water bath at 30 - 95 °C for 45 minutes. Once the desired reaction temperature is reached, TEOS (1.0×10^{-3} to 1.0 mol dm^{-3} TEOS) is rapidly added, then the reaction solution is aged at constant temperature (10 minutes to 24 hours) to form a uniform homogeneous layer of silica on the particles. During the aging process, adequate agitation such as stirring or bath sonication may be employed to ensure proper mixing and to eliminate particle agglomeration. After aging, the coated magnetic particles are repeatedly washed with distilled water until a neutral pH is reached. Between washing steps the coated particles are removed by an external magnetic field. The resulting coated particles are dried in a vacuum oven at 100 °C for 24 hours.

Other methods of producing completely sealed particles having an inorganic protective layer may be used in the present invention without departing from the scope and spirit of the present invention. The description above is not meant to limit the invention in any way. It is important that the protective layer completely seal and protect the monodisperse metallic core. Particles which are not completely coated will dissolve in 12 N HCl at room temperature in three minutes. In contrast, the completely sealed particles produced by the methodology of the present invention will not be effected by immersion in room temperature 12 N HCl for a period of one week.

The existence of and the completeness of sealing by the silica protective layer is verified by bathing the particles in acid environments. Un-coated iron oxide particles are dissolved by concentrated hydrochloric acid (such as 12 N HCl) almost instantaneously, yielding a yellow solution containing free iron. The dissolution rate is slowed down appreciably, or even stopped, if the iron oxide particles are coated. Therefore, the dissolution time of silica coated particles in 12 N HCl or other strong acid can be used to confirm and to evaluate the completeness of silica coating. For well coated composite iron oxide particles, the mean diameter of the particles increase after the coating, and the dissolution time in 12 N HCl increases significantly as well. A dissolution time in 12 N HCl greater than 1 hour generally indicates a relatively complete silica coating layer on the particles. As noted above, coated

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composite iron oxide particles according to the invention are remarkably stable in 12 N HCl more than 1 week.

The superior acid stability described above has never been achieved by any pre-existing particles, primarily because none of the magnetic particles in the prior art are sealed by inorganic rigid layers. Instead prior art particles are coated either by loosely bounded proteins or by polymers forming non-compact networks. The coated composite magnetic particles of the invention have many other useful properties, including their being base, salt and heat resistant, having excellent redispersibility in aqueous solutions, and having the ability to have chemical functional groups bound to the particle surfaces. By controlling the coating thickness, the overall density and magnetic susceptibility of the particles are controllable parameters. This is an alternative way to customize magnetic particles for specific applications, in addition to the previously described seed controlling method.

Coupling of Biological Ligands to Composite Magnetic Particles

A wide variety of methods for coupling various biological ligands to the coated particles are disclosed in the prior art. These same methods can be utilized with the particles of the invention. These methods generally involve either non-covalent adsorption and entrapment or covalent linkage. The first two approaches can not provide desirable biological ligand stability. Covalent coupling, an avidin-biotin-type linkage, is preferred for particles utilized in biological assay processes. The hydroxyl groups on the silica surfactant be converted to other functionalities to facilitate ligand attachment to the particle surface. For example, hydroxyl groups on the silica surface can be converted to highly reactive thiol groups and subsequently thiol groups can be converted to amines, carboxylic acids, etc.. The ability to introduce a wide variety of new functional groups to the inorganic surface layer, through the modification of the surface hydroxyl groups, provides for the coupling of wide range of biological materials.

A wide variety of processes for biological ligand attachment, conjugation, and cross-linking are well known in the art. [Weetall, *Meth. Enzymol.*, 44: 134 1976; J. F. Kennedy and J. M. S. Cabral, "Immobilized Enzymes in Solid Phase Biochemistry" W. H. Scouten, Ed., Wiley Interscience, New York, p. 253 (1983); W. Haller, "Application of Controlled Pore Glass in Solid Phase Biochemistry", in *Solid Phase Chemistry: Analytical and Synthetic Aspects,* W. H. Scouten Ed., Wiley Interscience, New York, Chap 11 (1983); H. H. Weetal, *Methods Enzymol.*, 44: 123 (1976); T. Miron and M. Wilchek, *Methods Enzymol.*, 135: 102 (1987); D.

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L. Wu, R. R. Waters, *J. Chromatog.*, 485: 169 (1988); P. Sudi, E. Dala, and B. Szajani, *Appl. Biochem. Biotechnol.*, 22: 31 (1989)]. Covalent attachment of biological ligands such as proteins, to a silica coated magnetic particle involves the formation of covalent bonds between reactive groups on the silica particle surface and the functional groups of the biological ligand. When binding proteins to the composite magnetic particles, several factors must be considered in order to retain the maximum biological activity of the protein. For example, proteins must be attached to the particle surface in an orientation that leaves the active sites or binding domains of the protein accessible to the substance desired to removed in a separation process to allow binding of the substance to the protein.

The coupling of biological ligands to the silica coated composite magnetic particles of the invention generally involves three steps: (1) derivatization of surface hydroxyl groups to other chemically active functionalities, such as thiols, amines and/or carboxylic acids, (2) activation of surface functional groups, and (3) coupling of biological ligands to the activated surface functional groups. The details of these steps are illustrated below.

Derivatization of Surface Functional Groups

Highly reactive carboxylic acid groups can be derivatized onto the silica surface through modification of silica surface hydroxyl groups. In a typical conversion process, silica coated magnetic particles (1.0 mg/ml to 50.0 mg/ml) are mixed with chloroacetic acid ($ClCH_2COOH$, 0.1M - 2.0M) in a aqueous methanol/water mixture (10% methanol-80% water) under agitation for certain period of time (5 - 60 minutes). Other useful reagents for the conversion of hydroxyl groups to of carboxylic acids include, but are not limited to, carboxylic acids such as acetic (CH_3COOH), iodoacetic (ICH_2COOH), 2-chlorobutanoic ($CH_3CH_2CHClCOOH$), 3-chlorobutanoic ($CH_3CHClCH_2COOH$), 4-chlorobutanoic ($ClCH_2CH_2CH_2COOH$), trichloroacetic ($Cl_33CCOOH$), trifluoroacetic (F_3CCOOH), and dicarboxylic acids such as oxalic acid (HO_2CCOOH), malonic acid (HO_2CCH_2COOH), succinic acid ($HO_2C(CH_2)_3COOH$, glutaric acid ($HO_2C(CH_2)_3COOH$, maleic acid (Z-$HO_2CCH{=}CHCOOH$), and fumaric acid (*E*-$HO_2CCH{=}CHCOOH$). The conversion reaction is repeated several times to ensure the complete conversation of the surface hydroxyl groups to carboxyl groups on the particles' surfaces. Between each conversion step, the magnetic particles are removed from the reaction solution by a magnetic field, washed and redispersed in a mixed methanol-water solution.

The presence of carboxylic acid groups can be confirmed by the observation that

antibodies can be attached to the particles using carboxyl group directed coupling agents. The molar amount of carboxylic acid on the particles' surface can be easily quantified using any standard antibody assays, such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA).

In an alternative embodiment, the relatively unreactive hydroxyl groups on the silica surface are converted to highly reactive amino groups by reaction with amine-containing organosilanes, such as 3-amino- propyltriethoxysilane, 4-aminobutyltriethiethoxysilane, 3-aminopropyl-diisopropyl- ethoxysilane, 3-amino- propylmethyldiethoxylsilane, N-(2-aminoethyl)-3-aminopropylmethyldimethoxysilane, triamino-functional silane ($H_2NCH_2CH_2$-NH-CH_2CH_2-NCH_2CH_2-CH_2-Si-$(OCH_3)_3$, 3-aminopropyltris(trimethylsiloxy)-silane, trimethoxysilylpropyldiethylenetriamine, and 1,3-bis(4-aminobutyl)tetramethyldisiloxane. The presence amino groups on the particles' surface can be confirmed, by radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

For certain applications, it is desirable to further convert amines to other functionalities. Such conversion can either increase the nucleophilicty or change the specificity of the original functional groups on the particles. After being derivatized to the particles, surface amino groups may be further converted to carboxylic acid or sulfhydryl groups, which provide means of tailoring the chemical reactivity of the silica surface of the particles to allow binding of a variety of biological materials to the particle surface.

The conversion of amine to carboxylic acid can be achieved by reacting amine derivatized composite magnetic particles with any of the following modifying reagents: dicarboxylic acid anhydrides, glacial acid anhydride, or succinyl chloride. Dicarboxylic acid anhydrides such succinic and maleic anhydrides, and glacial acid anhydride are preferably used in this invention. Both succinylation using succinic anhydride and maleylation using maleic anhydride can result in changing a cationic amino group to an anionic carboxylic acid.

According to the present invention, the carboxyl groups may also be derivatized to the silica surfaces of the particles by a silanization reactions similar to those described above, except that carboxyl-containing silane coupling agents such as N-[(3 trimethoxylsilyl)-propyl] ethylenediamine triacetic acid (CH_2-CH_2-CH_2-Si$(OMe)_3$-NH_2-NH_2-$(CH_2$-$COOH)_3$), 3-trimethylsilylpropionic acid ($Me_3SiCH_2CH_2COOH$), and N-[3-(triethoxysilyl)- propyl] phthalamic acid are used in place of the amine-containing organosilanes.

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For conjugating enzymes to the particles' surface (including, but not limited to alkaline phosphatase, horseradish peroxidase, β-D-galactosidase, glucose-6-phosphate dehydrogenase, acetylcholinesterase, inorganic pyrophosphates, lactoperoxidase, lactate dehydrogenase, glucoamylase, ribonuclease, and urease), the hydroxyl groups on the particle surface can be converted to free sulfhydryl groups. Thiolation reactions are preferred, namely, mercaptosuccinylation.

Mercaptosuccinylation is first carried out at room temperature by incubating amine-derivatized magnetic particles in a sodium phosphate buffer with a sufficient excess (10 - 500 fold) of a solution of S-acetylmercaptosuccinic anhydride in N, N-dimethylformamide for about 10 to 100 minutes. Hydroxylamine is then added to the mixture dropwise. Particles having free thiol groups are obtained after incubation at 30 °C for a short period of time (5 - 30 minutes). Excess chemical reactants are washed and thiol derivatized magnetic particles are redispersed with water or methanol, using an external magnetic field. Other methods of introducing thiol groups include using other converting agents, such as N-succinimidyl 3-(2-pyridyldithiol)propionate (SPSP), methyl 3-mercaptopropionimidate hydrochloride, 2-iminothiollane, and other thiol-containing succinimidyl derivatives.

Using chemical modifying agents, the interconversion among derivatized functional groups on the particle surfaces provides an additional means of diversifying the conjugation of biological materials. In one embodiment of this invention, carboxyl groups on silica coated magnetic particles can be converted to cationic amines, by reacting water-soluble carbodiimides such as 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC) or 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide (CMC) and diamine such as ethylenediamine at acidic conditions (pH 3.0 - 5.5). The thiol groups on composite magnetic particles may be converted to either amines or carboxylic acids depending on the type of biological ligand to be coupled. The conversion of thiols to amines can be carried out with ethylenimine and 2-bromoethylamine at slightly alkaline conditions. The conversion of thiols to carboxylic acids can be achieved through a carboxyalkylation reaction, using α-haloacetates as alkylating agents.

To attach most biological ligands, especially for coupling of proteins and enzymes, it is advantageous to have intermediate spacers between the coupling surfaces and the ligands to reduce steric hindrance. According to the present invention, the multiple derivatization or interconversion among surface functionalities may be utilized not only to tailor chemical reactivity on the magnetic particle surfaces specifically for coupling of an intended ligand, but also as an

effective vehicle for introducing chemical spacers on the particle surfaces. For example, the multiple derivatization of amine groups to carboxyl groups, and then reconverting the carboxyl groups to amine groups forms a 12-carbon spacer on silica surface of the magnetic particles.

The surface functional groups are highly stable because they are attached to the silica surfaces of magnetic particles primarily through covalent binding. This provides a significant advantage over those particles in the prior art, for example, the silane coated magnetic particles described by U.S. Pat. No. 4,695,393, where amine-containing organosilanes may be attached to iron oxide cores both covalently and adsorptively. The adsorbed polymerized organosilanes on the prior art particle surfaces may dissociate from their iron oxide cores during or after the ligand-coupling steps, which reduces the biological activity of the particles, and increases the non-specific binding of unwanted materials to vacant bare spots on the iron oxide core surface.

Activation of Surface Functional Groups And Protein Immobilization

Various approaches are available to immobilize biological materials onto the silica surfaces of the particles of the invention. The choice of chemical coupling method is dictated by the type of ligand to be coupled and the functionality on the surface of magnetic particle. In the case of proteins, many of the amino acid side chains of proteins are available for conjugation. For example, one of the most common groups used for attachment is the amino groups present on the N-terminus and the side chains of lysines. The carboxyl groups from the C-terminus, aspartic and glutamic acids, are also available for biding as well as the sulfhydryl groups of cysteines, hydroxyl groups of variety of reactive groups on the proteins. Thus, the type of protein immobilized to the particle surface is strongly dependent on the type of surface functionalities desired for coupling.

According to differing embodiments of the invention, one of four schemes may be employed to attach proteins onto the silica protective surface of the particles: (1) ligand immobilization by particle surface activation, (2) ligand immobilization using homobifunctional cross-linkers, (3) ligand immobilization using heterobifunctional cross-linkers, and (4) ligand immobilization through biotin-avidin linkage, described in detail later in the specification.

Within the framework of first scheme, attaching of biological ligands to the magnetic particles is typically carried out through the activation of amino group or carboxylic acid on the particle surface. Specifically, the amine terminated magnetic particles may be activated by cyanogen bromide, to which biological ligands comprising amino side groups, such as proteins, can be immobilized through guanidine linkages. Alternatively, surface amine activation of the magnetic particles may also be accomplished through a succinylation reaction followed by activation with

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N-hydroxysuccinimide. Amine-containing biological ligands such as proteins, enzymes, and avidins can then be covalently coupled to such an activated particle surface in known ways. Other useful amine activation approaches may include, but are not limit to, those using alkylation and acylation reactions with alkylating agents such as α-haloacetyl compounds, *N*-maleimide derivatives, aryl halides, aldehydes and ketones, and with acylating agents such as isocyanate, isothiocyanate, imidoesters, *N*-hydroxy-succinimidyl ester, *p*-nitrophenyl ester, acyl chloride, and sulfonyl chloride.

Of the various amine activation mechanisms, one particularly useful method involves the formation of Schiff bases on amine-terminated magnetic particles using carbonyl compounds such as aliphatic ketones and aldehydes. The reaction is pH dependent with best results obtained at basic conditions (pH 8 - 10). The adduct formed on the particle surfaces is reversible but can be stabilized using reducing agents such as sodium borohydride, sodium cyanoborohydride, or amine boranes such as dimethylamine, trimethylamine, t-butylamine, morpholine and pyridine boranes. An effective reduction process is typically carried out at 0 °C in a borate buffer (0.1M - 1.0M) of basic pH. Preferably, sodium cyanoborohydride is used to stabilize Schiff bases at low concentration (0.01M - 0.5M). A variety of amine-containing biological materials may be attached to the magnetic particle surfaces through Schiff base linkages.

According to one embodiment of the invention, the activation of carboxyl-terminated magnetic particles is accomplished utilizing a carbodiimide-mediated process. The carboxylic acid terminated magnetic particles are first washed and suspended in water. Under constant agitation, water soluble carbodiimide is added to the magnetic particles with a proper mass ratio between the particles and carbodiimide (5:1 - 50:1), to form *O*-acylisourea groups on the particle surface. Preferably, activated magnetic particles are washed several time before ligand attachment, to remove excess carbodiimide. Finally, the washed magnetic particles are suspended in water or biological buffers for subsequent coupling of biological ligands. Then, amine containing biological ligands can be attached to the particle surface by forming corresponding amide bonds. Any carbodiimide may be employed as an activation agent. However, two preferred water soluble carbodiimide derivatives are 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide (CMC) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). These two compounds can also be used to convert a carboxyl group to a cationic amine group.

As a useful alternative, surface carboxylate groups on magnetic particles may be activated first with *N*-hydroxylbenzotriazole in the presence of a water-soluble carbodiimide such as CMC or EDC to form reactive esters, which then are used to form amide bonds with amino groups of

biological ligands. Preferably, the ester-activated magnetic particles are washed extensively before the final coupling step to ensure removal of excess carbodiimides. *p*-nitrophenol and *N*-hydroxysuccinimide may also be used to form active esters on carboxyl-terminated magnetic particles, in the presence of carbodiimide. In another embodiment of the present invention, thionyl chloride may be used to activate carboxyl terminated magnetic particles to form acyl chloride on the particle surface. Proteins or enzymes are then immobilized onto the particle surface through the highly reactive acyl chlorides.

Homobifunctional and heterobifunctional cross-linking may also be employed to form covalent linkages between the surface functional groups on magnetic particles and the biological ligands. Homobifunctional reagents are generally those compound that contain two identical reactive functional groups which can introduce cross-linking intra-molecularly between two locales within a macromolecule and inter-molecularly between two molecules. Glutaraldehyde is the most prevalent homobifunctional reagent for the immobilization of proteins. [A. M. Klibamov, *Science*, 219:722 (1983)]. Heterobifunctional cross-linking reagents contain two dissimilar functional groups of different specificities, in any combination of group-selective moieties. For example, one end of the cross-linker may be selective for an amino group while the other end may be directed to a sulfhydryl group. This different reactivity of two different functional groups provides much needed flexibility to controlling the cross-linking selection and sequence.

During cross-linking, glutaraldehyde forms polymers in solution. At acidic pH, the polymers are cyclic hemiacetal. At neutral or slightly alkaline pH, the cross-linking occurs, and the α, β-unsaturated aldehyde polymers are formed and increase length as the pH is raised. Unsaturated aldehyde polymers cross-link the amino groups of the proteins with those on the surfaces of the particles. According to one embodiment of the present invention, the two-step cross-linking process is preferred, where the magnetic particles are first activated with glutaraldehyde, and then washed and coupled to the protein.

In carrying out this cross-linking process, amine terminated magnetic particles (1mg - 100 mg) are first washed several times with water or a biological buffer of low ionic strength, such as phosphate buffer (0.05M to 0.25M), before use. After washing, the magnetic particles are resuspended in a glutaraldehyde-containing (2% - 10% V/V), buffered aqueous medium at neutral pH (such as 0.1 M phosphate buffer of pH 7.5). The particles then react with glutaraldehyde at room temperature under gentle agitation for at least one hour, preferably three to five hours. After multiple washing steps, glutaraldehyde-activated particles are resuspended in a biological buffer

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at neutral or a slightly basic pH. At such pH values, bioaffinity of proteins can be preserved completely. The final protein coupling step is carried out by simply adding proteins to the glutaraldehyde-activated particles and mixing at room temperature for at least 5 hours, preferably more than 15 hours. Then the protein-conjugated magnetic particles are washed extensively with water or a buffer solution, to remove unbound proteins. Other glutaraldehyde derivatives may also be used to immobilize proteins. Two such derivatives are 3-methyl-glutaraldehyde and 3-methoxy-2, 4 dimethyl-glutaraldehyde.

In the present invention, protein immobilization may also be accomplished by utilizing formaldehyde, a very useful cross-linking reagent. A two-step protein coupling process is preferred. First, amine-terminated magnetic particles are mixed with formaldehyde, and surface amino groups are attacked by formaldehyde to form a quaternary ammonium salt, which can be subsequently converted to an immonium cation by losing a molecule of water. Such strongly electrophilic cations can react with a number of nucleophiles in the protein, producing a methylene-bridged cross-link. Before coupling to proteins, activated magnetic particles are preferably washed to remove excess reactants, which may interfere with the subsequent protein coupling process or even denature proteins. Then, the protein of interest is added and mixed with activated magnetic particles at room temperature for 5 to 15 hours. Unbound proteins are washed away with water or a buffer solution.

In another embodiment of this invention, proteins are immobilized onto hydroxyl-terminated magnetic particles by using several useful cross-linking reagents, including quinones such as *p*-benzoquinones, chloroforms and carbonyldiimidazole, heterocyclic halides such as cyanuric chloride and fluoropyrimide, bisoxiranes such as 1,4-butanediol diglycidyl ether (1.4-bis(2,3-epoxypropoxy)butane), and divinylsulfone. In the case of quinone for example, *p*-benzoquinone is mixed with magnetic particles for at least 3 hours, preferably 5 to 15 hours. After washing away excess quinone, proteins are then bound to the activated magnetic particles through their nucleophilic groups. This coupling reaction can be carried out in a broad pH range (from 3 to 10). The coupling schemes described above provide an advantage over those utilizing amines or carboxylic acids for protein immobilization, as these schemes start with hydroxyl-terminated solid matrix and no extra steps of surface modification are needed.

In one embodiment of the present invention, a third coupling scheme, involving heterobifunctional coupling agents, is used to immobilize the biological ligands. These heterobifunctional reagents generally comprise two distinctly different functionalities to provide

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a stable cross-linkage between the two different functionalities, one on particle surface and the other on the biological ligand to be coupled. A typical heterobifunctional coupling process involves reacting the cross-linker with functional group-terminated magnetic particles. The activated particles then react with biological ligands to complete the immobilization process. For example, by reacting amine-terminated magnetic particles with succinimidyl 6-maleimidocaproate, surface-bound maleimide functional groups are formed through amide linkages. Then, reactive maleimides can link sulfhydryl groups of proteins through thioether bonds. Other heterobifunctional reagents, including *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), *N*-γ-maleimidobutyryloxy succinimide ester, *N*-succinimidyl-(4-iodoacetyl)amino benzoate, and succinimidyl 4-(*p*-maleiodophenyl)-butyrate, may be used to conjugate proteins to magnetic particles.

Immobilization of Streptavidin

While chemical cross-linkers are used to form covalent bonds, there are some species which physically associate with proteins to form such a tight complex that the bond formation is essentially irreversible. The streptavidin-biotin complex is a well known example of such a system. Streptavidin is very useful in many applications. For example, streptavidin is a key component in many diagnostic and separation technologies, and has recently shown considerable promise in targeted drug/imaging agent delivery systems. [S. F. Rosebrough, *Nucl. Med. Biol.* 20: 575 (1993); P. Oehr, J. Westermann and H. J. Biersack, *J. Nucl. Med.* 29: 728 (1988); G. Paganelli, M. Malcovati and F. Fazio, *Nucl. Med. Commun.* 12: 211 (1990)].

Streptavidin is a biotin-binding protein found in the culture broth of the bacterium *streptomyces avidinii*. Like avidin, streptavidin binds 4 moles of biotin per mole of streptavidin with a high affinity virtually unmatched in a nature ($K_d = 10^{-15}$M). Streptavidin lacks carbohydrate side chains present on avidin and has an isoelectric point nearer to neutrality, where most useful biological interactions occur (pH of 5-6 *vs* 10 for avidin). As a result, streptavidin frequently exhibits lower levels of non-specific binding than does avidin when used in applications relying upon the formation of avidin-biotin complexes. [L. Chaiet and E. J. Wolf, *Arch. Biochem. Biophys.*, 108: 1 (1964); C. E. Agarana, I. D. Kuntz, S. Birken, R. Axel and C. R. Cantor, *Nucleic Acids Res.*, 14: 871 (1986); M. S. Brower, C. L. Brakel and K. Garry, *Analyt. Biochem.*, 147: 382 (1985); K. Hofmann, S. Wood, C. C. Brinton, J. A. Montbeller and F. M. Finn, *Proc. Natl. Acad. Sci.*, USA 77: 4666 (1980); G. Moxley, *Am. J. Clin. Path.*, 92: 630 (1989)].

Streptavidin has found utility in many biological applications, especially those using antibodies, because antibody molecules are easily modified by covalent attachment of biotin

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derivatives with little or no loss of the ability of the antibody molecules to bind their antigens. These biotinylated antibodies may be detected by their interaction with conjugates of avidin or streptavidin and the label enzymes. The same preparation of conjugated streptavidin/label enzyme may be used with any number of different biotinylated antibodies making such a system a highly flexible one. The label enzyme may be bound to streptavidin covalently, or biotinylated and attached to streptavidin via the streptavidin-biotin interaction. Since streptavidin is multivalent (binding 4 biotin molecules per tetrameric protein molecule), it may be used in combination with biotinylated label enzymes. ELISA systems employing streptavidin can readily detect sub-nanogram amounts of antigens.

As one embodiment of this invention, streptavidin coupled magnetic particles are made through a biotin-avidin linkage. Specifically, amine-terminated magnetic particles are first biotinylated using biotin derivatives, preferably those with chemical spacers such as biotinyl-ϵ-aminocaproic acid *N*-hydroxysuccinimide ester, sulfosuccinimidyl-6-[biotinamido] hexanoate, and [2-(biotinamido)ethylamidio-3,3'-dithiodipropionic acid *N*-hydroxysuccinimide ester, to reduce the stearic hindrance in binding streptavidin. A typical biotinylation reaction can be carried out in a bicarbonate buffer at basic pH (8.0 - 9.0). Biotin esters may be added directly to amine-terminated particles suspended in bicarbonate buffer. After being thoroughly mixed, the biotin-particle buffer solution is incubated at 0 °C for at lease 2 hours. Preferably, gentle agitation is applied during the biotinylation step to avoid particle settling. To remove unreacted biotin, the magnetic particles are repeatedly magnetically separated from the solution and then redispersed in water or the biological buffer. The final biotinylated magnetic particles can be resuspended in a buffed aqueous medium at neutral pH for subsequent streptavidin attachment.

The attachment of biotin on the magnetic particles and their streptavidin binding capacity can be confirmed and measured by absorbance measurements conducted on particles with enzyme-conjugated streptavidins such as streptavidin-horseradish peroxidase(HRP) and streptavidin fluorescein. A typical assay for measuring the biotin binding activity/capacity of the particles can be carried out by first mixing HRP-conjugated streptavidin with biotinylated magnetic particles in water or in a biological buffer of near neutral pH for at least 30 minutes. The molar amount of enzyme-conjugated streptavidin added to the testing sample is at least 10 times the maximum binding capacity of the biotinylated particles, based on theoretical calculations. The unbound streptavidin is washed away by repeated separation and redispersion of the magnetic particles. The washed particles are resuspended in an enzyme substrate such as ortho-phenylene-diamine (OPD)

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to generate color. The amount of bound streptavidin is readily determined by reading absorbance of particle/substrate mixture and comparing to the HRP-streptavidin standard.

Based on the measured biotin binding capacity, streptavidin, generally 5 to 50 times the binding capacity, can be added to biotinylated magnetic particles in the biological buffer to complete streptavidin immobilization. Preferably, a constant and gentle mixing is applied to eliminate possible particle aggregation and gravitational settling during the coupling step. The amount of streptavidin bound to the magnetic particles can be determined by the assay method described above, utilizing an enzyme-conjugated biotin, such as alkaline phosphatate-biotin, and horseradish peroxidase-biotin. As an alternative, streptavidins may be immobilized onto the magnetic particles through the coupling approaches described earlier. The amino- or carboxyl-termini of the polypeptide subunits on streptavidin may be utilized for immobilization. Preferably, chemical spacers are introduced in between the streptavidin and the solid surface of magnetic particles to minimize stearic hindrance. In accordance with the present invention, such spacers may be chemical compounds or biological molecules such as proteins.

A wide variety of biological ligands can be immobilized onto the streptavidin-coupled magnetic particles utilizing biotin-avidin linkages. Specifically, the biological ligand of interest such as an antibody, oligonucleotide and enzyme is first biotinylated and purified using standard biotinylation protocols. Then the biotinylated ligands can be coupled to streptavidin coated particles by simply mixing them in water or in a biological buffer at a near neutral pH. While streptavidin has shown utility in a wide variety of biological applications, the extremely high affinity of the streptavidin subunits for biotin, a necessity in many applications, can be detrimental in applications where reversible immobilization of streptavidin or biotinylated targets is ultimately desirable. For example, in an affinity based purification process to separate biotinylated target proteins, the ability to release and recycle the biotinylated target proteins and capture solid materials is not only desirable, but a requirement. In one embodiment of the present invention, generically engineered chimeric streptavidin tetramers [A. Chilkoti, B. L. Schwartz, R. D. Smith, C. J. Long and P. S. Stayton, *Biotechnology*, 13: 1198 (1995)] can be immobilized, directly or indirectly, onto the composite magnetic particles, to achieve reversible immobilization of target materials. Such genetically engineered, chimeric streptavidin tetramers are composed of subunits of both wild-type (WT) streptavidin and genetically-engineered streptavidin variants. The variant portion comprises the subunits of a site-directed mutant, Trp120Ala (W120A), which displays a biotin-binding constant about 10^4 times smaller than that of WT streptavidin. The vastly different

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rate kinetics of WT (K_a @ 1015 M^{-1}) and W120A (K_a @ 10^7 M^{-1}) subunits can effectively direct the biotinylated targets (magnetic particles) to high-affinity WT subunits, depending on which component is first coupled to the chimeric streptavidin tetramers. This new separation scheme combines advantages of both magnetic particles and duo biotin-avidin binding affinity of subunits of chimeric streptavidin tetramers, allowing irreversible immobilization of biotinylated targets (or biotin derivatized magnetic particles) at the higher affinity subunits, while retaining the reversible separation capabilities of lower affinity biotin derivatized magnetic particles (or biotinylated targets).

Binding of Oligonucleotides

Oligonucleotides are bound to various solid supports in many molecular biology-related applications, such as mRNA purification, solid phase cDNA synthesis, subtractive hybridization, solid phase DNA sequencing, and DNA/RNA binding protein isolation. Oligonucleotides can be attached to the composite magnetic particles of the invention through direct covalent-conjugation or through indirect biotin-streptavidin linkage. Preferably, the oligonucleotides are modified to introduce amine or carboxylic acid functionalities to the oligonucleotide. To covalently couple amine-modified oligonucleotides to the particles, a carbodiimide-mediated process is preferably used. Specifically, the carboxyl-terminated magnetic particles are first washed several times with water and resuspended in a biological buffer, such as 150 mM imidazole buffer at pH 7. Then, a known amount of the washed magnetic particles are incubated with a known amount of oligonucleotide, such as 5'-amino modified oligo $(dT)_{25}$ with a carbon spacer, in a freshly prepared coupling buffer, such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in imidazole buffer at pH 7, for at least 3 hours at a elevated temperature (35 - 75 °C). The mass ratio of the magnetic particles and the oligonucleotides should have the oligonucleotides in excess of the estimated binding capacity of the magnetic particles. To prevent particle aggregation and gravitational settling, gentle agitation is applied during the coupling reaction. After incubation, the magnetic particles are washed repeatedly with a RNAase-free washing buffer to remove unbound oligonucleotide.

Other covalent coupling methods, as described earlier, may also be employed to immobilize oligonucleotides. For example, the attachment of amine modified oligo $(dT)_{25}$ onto amine-terminated magnetic particles can be accomplished through glutaraldehyde activation of the functional groups. The oligonucleotides may also be immobilized on the streptavidin-coupled magnetic particles through biotin-streptavidin linkages. If biotin-streptavidin linkage is employed,

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biotin must be first introduced into the amine-derivatized oligonucleotides, such as 5'-amino modified oligo $(dT)_{25}$, before being coupled to the streptavidin-coupled magnetic particles. In this method, amino groups with chemical spacers are first introduced to the oligonucleotide, followed by covalent biotinylation of the amino groups. The biotinylated oligonucleotides can be coupled to the streptavidin-coupled magnetic particles by incubating them at room temperature for at least 30 minutes. Gentle agitation is generally preferred to avoid possible particle aggregation and gravitational settling. Finally, unbound excess oligonucleotides are removed by washing.

Many standard techniques can be used to measure the specific binding capacity of oligo-coupled magnetic particles. For example, the binding capacity of oligo $(dT)_{25}$- coupled magnetic particles can be determined easily by extracting poly(A)$^+$ RNA with decreasing amount of particles in parallel samples and measuring the amount of eluted RNA by wavelength scanning from 200 -- 300 nm or by standard Northern blotting.

Although it is well known in the art to bind biological materials to metallic or magnetic particles, prior art coated particles utilize organic or organo-silane coating as a substrate on which to bond the biological materials to the particles. As noted earlier, such organic coatings are unable to completely protect the magnetic particlesfromthe harsh environments encountered when the particles are utilized in biological separation processes. Either the prior art particles were incompletely coated, leaving the magnetic core exposed to the harsh environments immediately upon entry into the separation vessel, or the prior art coatings were oxidized by the harsh environments, eventually exposing the magnetic cores. The complete inorganic coating of the particles of the invention provides an improvement over the prior art particles with respect to the particles' ability to specifically bind biological materials to the particles' surface.

The complete inorganic coating provides a particle surface that is more uniform and has a neutral charge density. This results in a greater amount (per unit surface area of the particles) of the biological materials being bound to the particles' surface. As a result, the binding efficiency of the biological material being bound to the particles' surface is increased Each molecule of silica bound to the metal core provides a -OH binding site for the biological materials. In contrast, prior art organic coatings such as polyglutaraldehyde and proteins have far fewer binding sites per unit mass of coating material.

Following are examples of preferred embodiments of the particles according to the invention. The embodiments described below are in no way intended to limited the scope of the invention as defined in the claims attached hereto.

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Examples

Example 1: Synthesis of Hematite (α-Fe_2O_3) Seed Particles

Well defined hematite particle seeds were prepared by hydrothermal aging of an acidified aqueous solution of $FeCl_3$. A solution of 200 ml of a 0.02M aqueous $FeCl_3$ solution and of 0.001M HCl was first filtered through 0.45μ filters and then heated to 80°C in a tightly sealed one liter Pyrex bottle. The heated acidified iron salt solution then was placed inside an oven at 100°C for 24 hours. After aging, the solution was cooled to room temperature and the precipitant hematite particles were separated from the reaction solution by centrifugation at a speed of 5000 RPM for 30 minutes. The precipitant hematite particles were resuspended in water in an ultrasonic bath. The washing step was repeated six times to ensure the removal of chloride and ferric ions. The washed hematite particles were resuspended in water to create a solution having an iron concentration of 20 mg /ml (determined by a standard iron assay). The mean diameter of the resulting hematite particles was measured by a submicron particle analyzer (COULTER Model N4MD) to be 86.4 nm with the standard deviation of 4.5 nm.

Example 2: Synthesis of Silica (SiO_2) Seed Particles

A reaction solution containing 17 ml of ammonium hydroxide (ACS grade, NH_3 29.9%) and 15 ml of water in 170 ml of 2-propanol (HPLC grade, 99.9%) was filtered through a 0.22μ Nucleopore membrane. After brief mixing, the reaction solution was rapidly transferred to a tightly sealed one liter Pyrex bottle and placed in a constant-temperature water bath of 45°C for 20 minutes to ensure a thermal equilibrium. 0.4 ml of tetraethylorthosilicate (TEOS, 98% pure) was added, and the reaction solution was aged at 45°C for 45 minutes. The resulting silica particles were separated from the reaction solution a BüChi Rotavapor at 85°C and subsequently resuspended in methanol. The washing step was repeated six times. The final washed silica particles were dried in a oven at 100°C for 36 hours. The mean diameter of the resulting silica particles was measured by a submicron particle size analyzer (COULTER Model N4MD) to be 75.0 nm with a standard deviation of 6.0 nm. After measuring the particles diameters, the samples were resuspended in water in an ultrasonic bath.

Example 3: Synthesis of Composite Magnetite Cores

Example 3A: Composite iron oxide cores were prepared by first dissolving 9.04 g $FeSO_4 \cdot 7H_2O$ in 80 ml deionized water which was previously flushed with N_2 for 1 hour to remove O_2. Then, 9 ml of the 20 mg Fe/ml hematite seed particles (as prepared in Example 1), 10 ml of 0.5 M KOH and 10 ml of 2 M KNO_3, which were prepared with oxygen-free, deionized water, were

added to the ferrous salt solution and transferred to a 500 ml glass reaction vessel. The reaction vessel was closed with an air tight plastic lid having several inlets for inserting a nitrogen gas tube, a thermometer, and a drop funnel. After mixing the reaction solution, N_2 bubbling was continued for an additional five minutes to remove residual oxygen. The reaction vessel, containing a hydroxide precipitate, was placed into a 90°C constant temperature water bath and aged for 4 hours. The entire aging process was carried out without any disturbance. After aging, a resulting black magnetite precipitate was removed utilizing an external magnetic field, and repeatedly washed and resuspended with 300 ml of deionized water until a neutral pH was reached. Between each washing step the black magnetite precipitate was removed from the wash by an external magnetic field. The final composite magnetite cores were suspended in 300 ml deionized water.

Example 3B: An alternative method of preparing composite magnetite cores involves base precipitation. A mixed ferric and ferrous salt solution was prepared by dissolving 0.590 mg of $FeCl_2 \cdot 4H_2O$ and 1.605 g of $FeCl_3 \bullet 6H_2O$ in 500 ml of deionized water. After 15 minutes of mixing, the iron salt solution was filtered through a 0.22μ filter. Then 2.5 ml of the 20 mg Fe/ml hematite seed particles (as prepared in Example 1) were added to and mixed with the iron salt solution. Air was rigorously excluded from the iron salt/seed particle system by two hours of N_2 bubbling. The reaction mixture was then placed in a 75°C constant temperature water bath. Once the reaction system reached a uniform 75°C, 15 ml of ammonium hydroxide (ACS grade, 29.9% NH_3) was rapidly added to the reaction mixture. The basic mixture (pH 9.5 - 10.0) was mixed for 3 minutes with an overhead mixer and purged continually with nitrogen gas, during which black magnetite particles precipitated. The resulting composite magnetite cores were removed by an external magnetic field and then washed with 500 ml of deionized water and redispersed in aqueous solution. Typically, 7 to 10 wash cycles were enough to ensure the removal of all unreacted components, non magnetic or weekly magnetic materials. After the last wash, the composite magnetite cores were suspended in 25 ml deionized water.

Example 3C: the methods disclosed in Examples 3A and 3B can also be performed with silica seed particles (as prepared in Example 2) instead of hematite seed particles, to form composite magnetic cores according to the invention.

Example 4: Formation of Silica (SiO_2) Protection Layer

Comparative Example 4A, (without sonication): The coating process begins by a preparing a solution of 100 ml 2-propanol (HPLC grade, 99.9% pure), 8.75 ml of ammonium hydroxide (ACS grade, 29.9% NH_3), and 10 ml of composite magnetite particles (a total of 20 mg Fe, as

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prepared in Example 3B). This reaction solution was thoroughly mixed and transferred to a tightly screw-capped reaction vessel. The sealed reaction container was placed in a constant temperature water bath at 45°C. Upon reaching thermal equilibrium, 0.2 ml of tetraethylorthosilicate (TEOS, 98% pure) was added and mixed with the previously prepared reaction mixture under vigorous agitation. The reaction vessel was then aged at 45°C for 60 minutes without disturbance. It was observed that composite magnetic particles agglomerated and settled down to the bottom of reaction vessel shortly after the addition of TEOS and stayed agglomerated during the remaining aging process. After aging, the particles were washed 3 times with 200 ml deionized water and 5 times with 200 ml methanol. Between each washing step the resulting particles were removed from the wash by an external magnetic field and resuspended via vortex. The final silica-coated magnetic particles were suspended in 50 ml of a mixture of 10 % methanol (v/v) and 90% Ploy(ethylene glycol-400) demethacrylate ($H_2C=C(CH_3)CO(OCH_2CH_2)_nO_2CC(CH_3)=CH_2$, $n^{20}{}_D$ = 1.4660, $F_p > 110\,^0C$). Then the suspended silica coated particles were dehydrated, using a BüChi Rotavapor at a constant temperature of 100°C, where methanol and water were evaporated. The final coated particles were suspended in 10 ml of deionized water. The measured mean particle diameter was 2.5μ, and particle size distribution was found to be highly heterogeneous.

Example 4B, (with sonication): A reaction medium containing a mixture of 100 ml 2-propanol (HPLC grade, 99.9% pure), 8.75 ml ammonium hydroxide (ACS grade, 29.9% NH_3), and 10 ml composite magnetite particles (a total of 20 mg Fe, as prepared in Example 3B) was prepared and transferred to a screw-capped reaction vessel. The sealed reaction container was placed into a constant temperature ultrasonication water bath of 45°C to reach thermal equilibrium. Once a uniform temperature in the reaction vessel was reached, 0.2 ml of tetraethylorthosilicate (TEOS, 98% pure) was rapidly added to and mixed to the reaction medium. The reaction vessel was then aged at 45°C for 60 minutes under ultrasonication to keep the particles in dispersion. No apparent particle agglomeration or material settling was observed after the addition of TEOS during the aging process. Shortly after the addition of TEOS, the reaction solution became slightly turbid, indicating the formation of silica coating layers on the particles and individual silica particles as well. The turbidity of the reaction mixture increased as the reaction continued. After aging, the resulting particles were removed by an external magnetic field and were washed 3 times with 200 ml deionized water and 5 times with 200 ml methanol. Between each washing step the resulting particles were removed from the wash by an external magnetic field. Between each washing cycle, the coated composite magnetic particles were dispersed, using a vortex mixer. The final silica

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coated magnetic particles were suspended in 50 ml of a mixture of 10 % methanol (v/v) and 90% poly(ethylene glycol-400) demethacrylate ($H_2C=C(CH_3)CO(OCH_2CH_2)_n$-$O_2CC(CH_3)=CH_2$, n^{20}_D = 1.4660, $F_p > 110°C$). Then the suspended silica coated particles were dehydrated using a BüChi Rotavapor at a constant temperature of 100° C, during which methanol and water were evaporated. The final coated particles were suspended in 10 ml of deionized water. The measured mean particle diameter was 0.554μ with a narrow particle size distribution (<15%).

Example 4C, (multiple coating with sonication): 30 ml of deionized water was added to 10 ml of the micron sized coated composite magnetic particles prepared in Example 4A. The particle solution then was sonicated for 1 minute in a Fisher Model 550 Sonic dismembrator at a power level of 5.5 and a 0.1 second pulse on/off, using an overhead ultrasonicator with a 1/2" horn (5.5" in length, 1.5" in body diameter, and 0.5" in tip diameter). After sonication, the particles were magnetically separated from the reaction medium and washed 2 times with 100 ml deionized water and suspended in 10 ml deionized water after the final wash. The sonicated particles were then treated to a second coating process, by following the steps of Example 4B. The resulting two-step coated composite magnetic particles exhibited excellent dispensability in deionized water. The measured mean particle diameter was 0.472μ with a narrow particle size distribution (<15%).

Physical Characterization of Silica Coated Composite Magnetic Particles

Four important physical characteristics of magnetic particles according to the invention are the particle size, particle size distribution, gravitational settling time in aqueous solution, and magnetic separation rate under an external magnetic field. The particle size and size distribution were measured by a sub-micron particle analyzer with size distribution processor analysis and multiple scattering angle detection (COULTER Model N4MD). Such particle sizer is capable of analyzing emulsions, particles in suspension and molecules in solutions of known viscosity. The particle sizer measures particles having a diameter within the range of 0.003 to 3μ, and calculates the particle size and size distribution from the solution's diffusion coefficient by photon correlation spectroscopy. The sample used for particle size analysis was a 2.5 ml solution of silica coated composite magnetic particles, as prepared in Example 4B, in degassed deionized water with a concentration of 0.005 mg Fe/ml. The measured mean particle diameter is 0.554μ with a narrow standard deviation (less than 10%) of particle size distribution (with unimodel analysis, number of runs = 5, and auto-range each run).

The gravitational settling time is defined as the span of time during which absorbance or turbidity of the particle suspension decreases by 90%. 3ml of the silica coated composite magnetic

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particles, as prepared in Example 4B, in deionized water, at a concentration of 0.01 mg Fe/ml, was pipetted into a 4.5 ml polystyrene square cuvette with 4 clear sides, and placed into a UV-visible spectrophotometer. The absorbance of the sample at 550 nm was measured at time zero and every 30 minutes after that. The settling time of the particles in this example was found to be greater than 400 minutes. This remarkable settling rate should be compared to the settling times exhibited by prior art particles of similar diameters: (a) 5 minutes for the 10μ particles of Hersh and Yaverbaum [*Clin. Chem. Acta* 63: 69 (1975)]; (b) 1 minute for the 50-160μ particles of Robinson et al. [*Biotech. Bioeng.*, XV: 603 (1973]; and (c) 150 minutes for the 0.561μ particles of Whitehead et al. [U.S. Pat. 4,695,393].

The magnetic separation rate is defined as the percentage change in turgidity of the testing sample as particles are separated from the system by a magnetic field. The test samples were prepared by pipetting 2 ml of silica coated composite magnetic particles (prepared according to Example4B) solution (in deionized water; iron concentration, 0.05 mg /ml) into a 4.5 ml polystyrene square cuvette. Then one side of cuvette was placed against the square face of a permanent magnet with the magnetic field perpendicular to the face of the cuvette, to magnetically separate the particles from the sample. After time intervals of 1, 2, 3, and 6 minutes, the absorbance of the supernatant (at 550 nm) was measured using a UV-visible spectrophotometer.

The magnetic separation rate is calculated according to the following formula:

$$\text{Magnetic Separation Rate} = 100\% \times [1.0 - Ab(t) / Ab(0)],$$

where Ab(t) is the absorbance of supernatant at the separation time t, and Ab(0) is the absorbance of testing sample before the magnetic separation. The magnetic separation rate of silica coated magnetic particles according to the invention are compared with the separation rate of particles produced according to Whitehead et al. [U.S. Pat. 4,695,393] in Table 1.

TABLE 1

Magnetic Particles	Magnetic Separation Rate 1 min	2 min	3 min	6 min
Composite Magnetic Particles of the invention[1]	75%	>95%	100%	-
Prior art magnetic particles[2]	10%	75%	80%	100%

[1] Composite magnetic particles: mean diameter = 0.557μ prepared according to Example 4B.
[2] Magnetic particles (0.56μ): according to Whitehead et. al., U.S. Pat. 4,695,393.

Although the two particles listed above have comparable particle mean diameters (0.557μ vs.

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0.60μ), the above results clearly indicate clearly the advantages exhibited by particles prepared according to the invention. the particles according to the invention maintain discrete and non-agglomerated in solution longer, while at the same time exhibiting much stronger magnetic behavior than prior art particles.

Chemical Stability Tests of Silica Coated Composite Magnetic Particles

This example discloses the superior chemical stability of silica coated magnetic particles according to the invention when the particles are placed in extreme environmental conditions, such as strongly acidic solutions, high ionic strength solutions, and high temperature. In each test two physical characteristics, particle size and magnetic separation rate, are measured before and after the particles undergo the testing condition. The test of the stability of the particles against strong acid was carried out by pipetting 50μl of a solution of silica coated composite magnetic particles, as prepared in Example 4B, (in deionized water at a concentration of 0.5 mg Fe/ml) into 200μl of 12 N HCl in a glass test tube, and recording the time at which the color of the testing solution turned yellow, indicating that iron oxidefromthe core of the particles was dissolved by the HCl. The same test was performed for bare magnetite particles (as prepared in Example 3B, without a silica coating) and the Whitehead particles (Promega, Streptavidin Magnetic Particles). Silica coated composite magnetic particles according to the invention were stable in 12 N HCl for more than 45 minutes, while the bare magnetite particles and the Whitehead streptavidin particles were dissolved by 12 N HCl in less than 30 seconds.

The stability of the silica coated composite magnetic particles according to the invention in high ionic strength media was tested by mixing 100 μl of a solution of silica coated composite magnetic particles, as prepared in Example 4B, (in deionized water, at a concentration of 0.1 mg Fe/ml) with 3 ml of 3 M NaCl. After 30 minutes, the particle size and its magnetic separation rate were measured, and compared with the results described in Table 1. Although the mean diameter for the particles in 3 M NaCl was 0.60μ, 7.7% larger than the diameter of the silica coated composite magnetic particles in deionized water, the magnetic separation rates were about the same as the results reported in Table 1, indicating that the particles according to the invention maintain their remarkable magnetic strength, even when placed in highly ionic media.

The thermal stability was tested by autoclaving a 20 ml sample of a solution of silica coated composite magnetic particles produced according to Example 4B (in deionized water at a concentration of 0.1 mg Fe/ml) at 121 °C using a laboratory sterilizer for 45 minutes. After cooling to room temperature, the mean particle diameter and magnetic separation rate were measured. No

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changes were found with respect to these two parameters, when comparing to the values in Table 1. A similar sample solution was frozen at -10°C for 12 hours. The particle size and magnetic separation rate were determined after the sample was defrosted and thermally equilibrated to room temperature. The results were same as the those presented in Table 1.

Example 5: Preparation of Amine-Terminated Composite Magnetic Particles

Silica coated composite magnetic particles, prepared according to Example 4B, in 100 ml deionized water (1.0 mg Fe/ml) were washed 3 times with methanol, using a magnetic field to separate the particles between washings. The washed particles were suspended in a 100 ml solution containing 95% methanol and 5% deionized water after the final wash cycle. The pH value of the particle suspension was adjusted to 4.5 with glacial acetic acid (99.9% pure, ACS plus grade), and the acidified particle suspension was placed into an ultrasonication bath (Fisher, Model FS14H) for 5 minutes. 1.0 ml of 3-aminopropyltriethoxysilane (CAS No. A0750, United Chemical Technologies, Inc.) was added rapidly into the particle solution with stirring. The reaction mixture was then poured into a 500 ml glass beaker and mixed for 2 hours with an overhead mixer at room temperature. After mixing, the amine-terminated particles were washed 2 times with 200 ml methanol and 5 times with 200 ml deionized water to remove unbound organosilane materials. Between each washing step the particles were removed from the suspension by an external magnetic field. The attachment of amine functionality on the particles surface was confirmed and quantified indirectly by subsequent biotinylation and measuring the biotin capacity.

Example 6: Attachment of Biotin to Amine-Terminated Particles

A 20 ml sample (1.0 mg Fe/ml) of amine-terminated composite magnetic particles, as prepared In Example 5, in deionized water were washed 3 times with 100 ml of 50 mM bicarbonate buffer, pH 8.5. Between each washing step the resulting particles were removed from the wash by an external magnetic field. After the final wash, the particles were resuspended in 20 ml of 50 mM bicarbonate buffer, pH 8.5, and transferred into a 60 ml clear glass, narrow-mouthed, round bottle with a cap. 1.0 mg of biotinamidocaproate N-hydroxy-succinimide ester was added directly to the particle solution under vortex mixing. The glass bottle containing the reaction mixture was then mounted on a orbital shaker, which was placed inside a refrigerator at 5°C. The total reaction time of biotinylation was 3 hours, during which a gentle mixing was applied by the orbital shaker. To remove unreacted biotin, the washing technique with intermediate magnetic extraction, as described above, was utilized. The biotinylated magnetic particles were washed 5 times with 100 ml of deionized water, and 2 times with 100 ml of 25 mM phosphate buffer, pH 7.2. After the final

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washing cycle, the particles were suspended in 20 ml of 25 mM phosphate buffer, pH 7.2.

Measurement of Biotin Biding Capacity Using HRP-Streptavidin

To determine the binding capacity of the biotinylated magnetic particles produced according to this Example, a HRP-streptavidin based assay system was utilized. 50 mg of lyophilized HRP-streptavidin powder (Sigma, product number S9415) was dissolved in 20 ml of 25 mM phosphate buffer, pH 7.2, and filtered through a 0.22μ filter. The final concentration of HRP-streptavidin solution was adjusted to 1.65μg /ml, based on the extinction coefficient, $E^{1\%}$ (280 nm) of HRP-streptavidin. The streptavidin binding buffer contains 25 mM phosphate at pH 7.2. The HRP substrate buffer was prepared by dissolving 10 mg ortho-phenylene in a solution of 25 ml 0.1M citric/phosphate buffer, pH 5.0, and 10μl of 30% H_2O_2. The particle-buffer solution was used within 15 minutes of preparation. The wash buffer was deionized water. A standard curve for HRP-streptavidin absorbance was first determined by measuring the absorbencies at 490 nm for 7 x 2 duplicated samples containing 0, 1.5, 5.0, 10, 20, 50, and 100 ng of HRP-streptavidin in 150μl of deionized water and 100μl of HRP substrate buffer. After 5 minutes incubation at room temperature, 100μl of 1 M H_2SO_4 was added to stop color generation, and all samples were transferred to a 96-well polystyrene microplate for absorbance reading, using a microplate reader (Model MRX, Dynatech Laboratories).

The binding assay was performed on 10 x 2 duplicated samples (containing 0.0, 0.05, 0.1, 0.2 , 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 μg Fe) of biotinylated composite magnetic particles suspended in 100μl of binding buffer in micro-centrifuge tubes. 100μl of 1.65 μg /ml HRP-streptavidin was added to and mixed with each testing sample. After 15 minutes incubation at 37°C, the testing samples were washed 4 times with 200μl wash buffer to remove unbound HRP-streptavidin. The particles were separated and redispersed by a 0.5" x 0.5" x 0.5" magnet. Three minutes of magnetic separation time was employed in this example, which is long enough to ensure a complete bound/free separation. The washed magnetic particles, coupled with HRP-streptavidin, were resuspended in 150μl deionized water. After adding 100μl of HRP-substrate buffer into each sample, a red-orange color of different intensity appeared in each testing sample within 4 minutes. Then 100 μl H_2SO_4 was added to stop color generation, and all samples were transferred to a 96-well polystyrene microplate for absorbance reading, using a microplate reader (Model MRX, Dynatech Laboratories).

Since iron oxide particles (with or without any coating materials) can absorb light of a broad range of wavelengths, the absorbance attributed to magnetic particles must be determined

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and subtracted from the total absorbance of a testing sample containing magnetic particles. Thus, the same assay method reported above was performed using amine-terminated composite magnetic particles as a control group, instead of using biotinylated magnetic particles. The absorbance reading of a testing sample is the cumulative absorbancefromtwo sources. One is the absorbance due to iron oxide particles, and the other is due to non-specific binding of HRP-streptavidin to amine-terminated particles. By subtracting the background and non-specific absorbance measurement from the absorbance measurement of a biotinylated particle sample containing same the amount of magnetic materials, the biotin binding capacity was determined. The result was that a remarkable 52.5 mg streptavidin were bound to a 1.0 g Fe equivalent of biotinylated magnetic particles.

Example 7: Attachment of Streptavidin to Biotinylated Particles

A binding buffer of 25 mM phosphate buffer, pH 7.2, and a washing buffer of 20 ml HEPES, pH 7.2, and a storage buffer of 20 ml HEPES/1% BSA, pH 7.2, (1 liter each) were prepared. Then 10 ml of a (1.0 mg Fe/ml) solution of biotinylated composite magnetic particles, as prepared in example 6, was washed twice with 20 ml of the binding buffer, and resuspended in 5 ml of the binding buffer. A stock solution of 7 mg of streptavidin (SA10 000, ProZyme, CA) dissolved in 10 ml of binding buffer was prepared and passed through a 0.22μ filter. The concentration of the filtered solution was determined according to the extinction coefficient of streptavidin ($E^{1\%}$ [@280 nm] = 32) before coupling. A sample of the stock solution containing a 5.5 mg equivalent of streptavidin was prepared for use in a reaction with the 10 ml of biotinylated composite magnetic particle solution.

Since the measured binding capacity of the biotinylated particles was 52.5 μg streptavidin per mg Fe equivalent of particles, the 5.5 mg equivalent of streptavidin added to the stock solution was 10 times the binding capacity of the 10 mg Fe equivalent of biotinylated composite magnetic particles in the binding buffer. The 10 ml sample of the biotinylated composite magnetic particle solution was added dropwise to 5.5 mg equivalent streptavidin solution, and the mixture was stirred with a magnetic stirrer at room temperature for 2 hours. The streptavidin coupled particles were washed 5 times with 25 ml washing buffer by separating and redispersing the magnetic particles, using an external magnetic field. After the removal of unbound streptavidin, the coupled particles were suspended in 10 ml of a storage buffer.

Example 8: Immobilization of Protein A

The immobilization of protein A to amine-terminated composite magnetic particles was

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carried out by a two-step homobifunctional cross-linking process using glutaraldehyde as activation agent. A dispersion of amine-terminated composite magnetic particles, as prepared in example 5, containing an equivalent of 100 mg Fe in deionized water, was washed 2 times with 200 ml of 50 mM phosphate buffer, pH 7.2, removed from the wash solutions using a external magnetic field, and resuspended in 100 ml of 50 mM phosphate buffer. A 3% (V/V) glutaraldehyde solution was made by diluting 25% glutaraldehyde stock solution (Sigma, Product Number G 5882) with 50 mM phosphate buffer. A 25 ml sample of the 3% glutaraldehyde solution was then added to the particle solution. The particles reacted with glutaraldehyde under gentle mixing with a magnetic stirrer for 3 hours at room temperature. The glutaraldehyde-activated particles were then removed from the reaction and washed five times, using an external magnetic field to remove the particles between washing steps, was employed to remove unreacted glutaraldehyde from the glutaraldehyde-activated particles. During each of the wash cycles, the particles were separated magnetically and redispersed with fresh 200 ml of 50 mM phosphate buffer, pH 7.2. After the final wash cycle, the glutaraldehyde-activated particles were resuspended in 50 ml of the phosphate buffer.

A protein solution of protein A was made by dissolving 65 mg of protein A (FC20 010 ProZyme, CA) in 50 ml of 50 mM phosphate buffer, pH 7.2. The protein solution was filtered through a 0.22μ filter, and its final concentration was determined based on the extinction coefficient of protein A ($E^{1\%}$ [@280 nm] = 1.4). Then, 50 ml of the glutaraldehyde-activated particle suspension was added dropwise into 45 ml of the protein solution (containing approximately 55 mg of protein). The reaction vessel was aged at room temperature for 15 hours under gentle agitation with an orbital shaker. The protein A-coupled particles were then washed 5 times magnetically with 100 ml of 25 mM phosphate buffer, pH 7.2, to remove unbound proteins. The washed protein A-coupled particles were finally suspended in 50 ml 25 ml HEPES, pH 7.5.

Example 9: Immobilization of Oligo $(dT)_{25}$

Example 9A, (carboxylation of amine-terminated particles): A carbodiimide-mediated process was employed to immobilize amine-modified Oligo $(dT)_{25}$ to carboxyl-terminated composite magnetic particles. First, the amine groups on the amine-terminated composite magnetic particles to were converted to acid groups. A 10 mg sample of the amine-terminated composite magnetic particles (as prepared in Example 5) were washed five times with 50 ml of 50 mM of $NaHCO_3$ using an external magnetic field to remove the particles between washes. The washed amine-terminated particles were then suspended in 20 ml of 50 mM sodium bicarbonate to which

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10 mg of glutaric anhydride was added. The suspension was allowed to react for 1 hour. After reaction, carboxyl -terminated particles were washed magnetically five times with 50 ml of deionized water, two times with 50 ml of 150 mM imidazole buffer, pH 7.0, and stored in 10 ml of 150 ml imidazole buffer.

Example 9B, (Oligonucleotide coupling to the particle surface): Oligo nucleotide $(dT)_{25}$ coupling was carried out as follows. A 10 ml sample of the of carboxyl-terminated particle suspension prepared in Example 9A was incubated with 0.4 mg of 5'-amino-oligo nucleotide $(dT)_{25}$ with a 20 carbon spacer (Customer synthesized) in 1 ml of freshly prepared coupling buffer (75 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 150 mM imidazole buffer, pH 7.3) for 5 hours at 45°C. Gentle mixing was applied during the incubation by mounting the reaction container (50 ml centrifuge tube) on an orbital shaker. After incubation, the magnetic particles were separated with a strong magnet, the supernatant was aspirated, and the particles were washed 5 times with 50 ml RNase-free deionized water (0.1% DEPC treated) at 60 °C and 3 times with 50 ml of 25 mM phosphate buffered saline (PBS). The particles were magnetically removed from the solution in between washes. The particles were resuspended in 10 ml of 25 mM PBS, pH 7.5, containing 0.1% Bovine Serum Albumin (BSA) and 0.02% NaN_3 and stored in 4 °C.

Example 10: Non Specific Binding (NSB) of Human White Blood Cells Using Streptavidin Particles

One of the most important features of bio-activated composite magnetic particles according to the invention, when used in cell biology applications, is their remarkable non-specific binding level. The non-specific binding level of the composite magnetic particles is an indication of how well the particles will perform in a separation system, namely the composite magnetic particles' ability to select and bind with specific target cells in the separation process in order to remove the target cells from the separation medium, especially when the population of target cells is extremely low. Whole human blood (WB) was used in this experiment as a system that is exemplary of most of human cell-related applications. The streptavidin-coupled magnetic particles prepared in Example 7 were used to capture white blood cells without using any antibodies against human cell markers as a method of determining the non-specific binding level of the particles.

An isotonic cell working buffer was prepared by dissolving 0.965 mg monosodium phosphate dibasic, 18.61 g EDTA, 1.0 g sodium azide, and 2.625 g sodium chloride in 1.0 liter of deionized water. 10 g BSA was also added to and dissolved in this cell working buffer. The pH was adjusted to 7.5 with 10 M NaOH. The osmolarity of this isotonic cell working buffer was 278

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mOsm/kg. A 0.5 ml batch of streptavidin-bound particles, containing a 0.75μg Fe equivalent, was mixed in the isotonic cell working buffer and incubated with 1 ml whole blood for 20 minutes at room temperature. The cells captured non-specifically by the streptavidin-bound particles were isolated from whole blood after 20 minutes by magnetic separation using a strong bar magnet. The supernatant was removed and the particles/captured white blood cells were resuspended in 1.5 ml of the cell working buffer. After 10 minutes, magnetic separation was employed to further remove any non-magnetic components. The particles/captured white blood cells were then resuspended in 150μl of the cell working buffer.

A control sample was prepared by mixing 10 μl of a diluted blood solution (10μl WB + 90μl cell working buffer) with 10μl of a counting dye, and was placed on a hemocytometer to count the white blood cells in the control sample. The amount of white blood cells in the particle/captured white blood cells was counted in a similar way (hemocytometer analysis of a solution comprising 10μl particle/captured white blood cell suspension+ 10μl counting dye). It was found that 205 white blood cell were captured by streptavidin particles from 1 ml whole blood which contained 5,935,000 white blood cells. Thus the non specific binding percentage (NSB) was calculated as a remarkable 0.00345% (100% x (205 / 5,935,000)).

Although this invention has been described in connection with specific form thereof, it is to be understood and appreciated that a wide array of equivalents may be substituted for the specific elements shown and described herein without departing from the scope and spirit of the invention as described in the appended claims.

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We claim:

1. A metallic particle comprising a metallic core coated by an inorganic material, wherein said coated particle has a diameter between about 0.03 and about 2.0μ.

2. The particle of claim 1, wherein said metallic core comprises a metallic oxide material.

3. The particle of claim 2, wherein said metallic oxide material comprises a metal portion chosen from the group consisting of Fe, Mn, Mg, Co, Ni, Zn, Cu, Al, Ti, Mb, Mo, Pd, Ag, Cd, Gd, Tb, Dy, Er, Tm, Hg, V, In.

4. The particle of claim 1, wherein said metallic core comprises magnetic material.

5. The particle of claim 4 wherein said magnetic material is chosen from the group consisting of magnetic metals and magnetic metal oxides.

6. The particle of claim 5, wherein said magnetic metals are chosen from the group consisting of Fe, Zn, Ni, and Co.

7. The particle of claim 5, wherein said magnetic metal oxides comprise a metal portion chosen from the group consisting of the oxides of divalent transition metal ions.

8. The particle of claim 7, wherein said divalent transition metal ions are chosen from the group consisting of Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2-}, Cu^{2-}, and Fe^{2+}.

9. The particle of claim 3, wherein said metal portion comprises a metal ion chosen from the group consisting of the following metal ions: Al^{3+}, Ti^{4+}, V^{3+}, Mb^{2+}, Co^{2+}, Ni^{2+}, Mo^{5+}, Pd^{3+}, Ag^{+}, Cd, Gd^{3+}, Tb^{3+}, Dy^{3+}, Er^{3+}, Tm^{3+}, Hg^{+} Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2-}, Fe^{3-}, and Fe^{2+}.

10. The particle of claim 2, wherein said metal oxides are chosen from the group consisting of ferric, ferrous, zinc, nickel or cobalt oxides.

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11. The particle of claim 2, wherein said metal oxide is magnetite.

12. The particle of claim 1, wherein said inorganic material is chosen from the group consisting of crystalline or non-crystalline silica (SiO_2) and other silica derivatives.

13. The particle of claim 1, wherein said coating material is formed from an inorganic silicate of the formula SiX_n, wherein X is a hydrolyzable group, chosen from the group consisting of alkoxy, acyloxy, amine, or chlorine.

14. The particle of claim 1, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least seven minutes without reaction of the metallic core and the HCl.

15. The particle of claim 14, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least one hour without reaction of the metallic core and the HCl.

16. The particle of claim 14, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least one week without reaction of the metallic core and the HCl.

17. The particle of claim 4, wherein said inorganic material is chosen from the group consisting of crystalline or non-crystalline silica (SiO_2) and other silica derivatives.

18. The particle of claim 4, wherein said coating material is formed from an inorganic silicate of the formula SiX_n, wherein X is a hydrolyzable group, chosen from the group consisting of alkoxy, acyloxy, amine, or chlorine.

19. The particle of claim 4, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least seven minutes without reaction of the metallic core and the HCl.

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20. The particle of claim 19, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least one hour without reaction of the metallic core and the HCl.

21. The particle of claim 19, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least one week without reaction of the metallic core and the HCl.

22. The particle of claim 11, wherein said inorganic material is chosen from the group consisting of crystalline or non-crystalline silica (SiO_2) and other silica derivatives.

23. The particle of claim 11, wherein said coating material is formed from an inorganic silicate of the formula SiX_n, wherein X is a hydrolyzable group, chosen from the group consisting of alkoxy, acyloxy, amine, or chlorine.

24. The particle of claim 11, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least seven minutes without reaction of the metallic core and the HCl.

25. The particle of claim 24, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least one hour without reaction of the metallic core and the HCl.

26. The particle of claim 24, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least one week without reaction of the metallic core and the HCl.

27. The particle of claim 1, wherein the batch of said particles are produced in a monodisperse size range.

28. The particles of claim 1, wherein the standard deviation of the size distribution of

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a batch of said particles is less than or equal to thirty-five percent of the mean diameter of the particles in said batch.

29. The particle of claim 4, wherein the batch of said particles are produced in a monodisperse size range.

30. The particles of claim 4, wherein the standard deviation of the size distribution of a batch of said particles is less than or equal to thirty-five percent of the mean diameter of the particles in said batch.

31. A metallic particle comprising a metallic core coated by an inorganic material, wherein said metallic core comprises a metal oxide material and an inorganic seed material, and said coated particle has a diameter between about 0.03 and about 2.0μ.

32. The particle of claim 31, wherein said inorganic seed material is chosen from the group consisting of metal hydrous oxides metals or silica.

33. The particle of claim 32, wherein said metal hydrous oxide material comprises a metal portion chosen from the group consisting of Fe, Mn, Mg, Co, Ni, Zn, Cu, Al, Ti, Mb, Mo, Pd, Ag, Cd, Gd, Tb, Dy, Er, Tm, Hg, V, In.

34. The particle of claim 31, wherein said metallic core comprises magnetic material.

35. The particle of claim 34 wherein said magnetic material is chosen from the group consisting of magnetic metals and magnetic metal oxides.

36. The particle of claim 35, wherein said magnetic metals are chosen from the group consisting of Fe, Zn, Ni, and Co.

37. The particle of claim 35, wherein said magnetic metal oxides comprise a metal portion chosen from the group consisting of the oxides of divalent transition metal ions.

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38. The particle of claim 37, wherein said divalent transition metal ions are chosen from the group consisting of Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+}.

39. The particle of claim 32, wherein said metal oxide comprises a metal ion chosen from the group consisting of the following metal ions: Al^{3+}, Ti^{4+}, V^{3+}, Mb^{2+}, Co^{2+}, Ni^{2+}, Mo^{5+}, Pd^{3+}, Ag^{+}, Cd, Gd^{3+}, Tb^{3+}, Dy^{3+}, Er^{3+}, Tm^{3+}, Hg^{+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}, and Fe^{2+}.

40. The particle of claim 32, wherein said metal oxides are chosen from the group consisting of ferric, ferrous, zinc, nickel or cobalt oxides.

41. The particle of claim 32, wherein said metal oxide is magnetite.

42. The particle of claim 31, wherein said inorganic material is chosen from the group consisting of crystalline or non-crystalline silica (SiO_2) and other silica derivatives.

43. The particle of claim 31, wherein said coating material is formed from an inorganic silicate of the formula SiX_n, wherein X is a hydrolyzable group, chosen from the group consisting of alkoxy, acyloxy, amine, or chlorine.

44. The particle of claim 31, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least seven minutes without reaction of the metallic core and the HCl.

45. The particle of claim 44, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least one hour without reaction of the metallic core and the HCl.

46. The particle of claim 44, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least one week without reaction of the metallic core and the HCl.

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47. The particle of claim 34, wherein said inorganic material is chosen from the group consisting of crystalline or non-crystalline silica (SiO_2) and other silica derivatives.

48. The particle of claim 34, wherein said coating material is formed from an inorganic silicate of the formula SiX_n, wherein X is a hydrolyzable group, chosen from the group consisting of alkoxy, acyloxy, amine, or chlorine.

49. The particle of claim 34, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least seven minutes without reaction of the metallic core and the HCl.

50. The particle of claim 49, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least one hour without reaction of the metallic core and the HCl.

51. The particle of claim 49, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least one week without reaction of the metallic core and the HCl.

52. The particle of claim 41, wherein said inorganic material is chosen from the group consisting of crystalline or non-crystalline silica (SiO_2) and other silica derivatives.

53. The particle of claim 41, wherein said coating material is formed from an inorganic silicate of the formula SiX_n, wherein X is a hydrolyzable group, chosen from the group consisting of alkoxy, acyloxy, amine, or chlorine.

54. The particle of claim 41, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least seven minutes without reaction of the metallic core and the HCl.

55. The particle of claim 54, wherein said particle is able to withstand placement in 12

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N HCl at room temperature for a period of at least one hour without reaction of the metallic core and the HCl.

56. The particle of claim 54, wherein said particle is able to withstand placement in 12 N HCl at room temperature for a period of at least one week without reaction of the metallic core and the HCl.

57. The particle of claim 31, wherein the batch of said particles are produced in a monodisperse size range.

58. The particles of claim 31, wherein the standard deviation of the size distribution of a batch of said particles is less than or equal to thirty-five percent of the mean diameter of the particles in said batch.

59. The particle of claim 34, wherein the batch of said particles are produced in a monodisperse size range.

60. The particles of claim 34, wherein the standard deviation of the size distribution of a batch of said particles is less than or equal to thirty-five percent of the mean diameter of the particles in said batch.

61. The particle of claim 1, wherein the inorganic coating material has on the external surface thereof hydroxyl groups.

62. The particle of claim 61 wherein the surface hydroxyl groups have been converted to organic functionalities, said functionalities chosen from the group consisting of thiols, amines, and carboxylic acids.

63. The particle of claim 62 wherein the surface hydroxyl groups have been converted to carboxylic acids by mixing the particles of claim 1 with a carboxylic acid in an aqueous methanol/water mixture (10% methanol-80% water) under agitation for period of 5 - 60 minutes.

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64. The particle of claim 63 wherein the carboxylic acid is chosen from the group consisting of chloroacetic acid ($ClCH_2COOH$), acetic (CH_3COOH), iodoacetic (ICH_2COOH), 2-chlorobutanoic ($CH_3CH_2CHClCOOH$), 3-chlorobutanoic ($CH_3CHClCH_2COOH$), 4-chlorobutanoic ($ClCH_2CH_2CH_2COOH$), trichloroacetic ($Cl_33CCOOH$), trifluoroacetic (F_3CCOOH), oxalic acid (HO_2CCOOH), malonic acid (HO_2CCH_2COOH), succinic acid ($HO_2C(CH_2)_3COOH$, glutaric acid ($HO_2C(CH_2)_3COOH$, maleic acid (Z-HO_2CCH=$CHCOOH$), and fumaric acid (*E*-HO_2CCH=$CHCOOH$).

65. The particles of claim 62 wherein the surface hydroxyl groups have been converted to amines by mixing the particles of claim 1 and amine-containing organo-silane monomer in a acidified solution at a temperature of 35 -60 °C and washing and dehydrating the particles.

66. The particles of claim 65 wherein the amine-containing organo-silane monomer is chosen from the group consisting of 3-amino- propyltriethoxysilane, 4-aminobutyltriethiethoxysilane, 3-aminopropyl-diisopropyl- ethoxysilane, 3-amino-propylmethyldiethoxylsilane, N-(2-aminoethyl)-3-aminopropylmethyldimethoxysilane, triamino-functional silane ($H_2NCH_2CH_2$-NH-CH_2CH_2-NCH_2CH_2-CH_2-Si-$(OCH_3)_3$, 3-aminopropyltris(trimethylsiloxy)-silane, trimethoxysilylpropyldiethylenetriamine, and 1,3-bis(4-aminobutyl)tetramethyldisiloxane.

67. The particles of claim 62 wherein the surface hydroxyl groups have been converted to thiol groups by incubating the particles of claim 65 in a sodium phosphate buffer with a sufficient excess (10-500 fold) of a solution of a thiolating agent in N, N-dimethylformamide, for about 10 to 100 minutes, and then adding hydroxylamine in a dropwise fashion.

68. The particles of claim 67 wherein the thiolating agent is chosen from the group consisting of S-acetylmercaptosuccinic anhydride, N-succinimidyl 3-(2-pyridyldithiol)propionate (SPSP), methyl 3-mercaptopropionimidate hydrochloride, 2-iminothiollane.

69. The particle of claim 31, wherein the inorganic coating material has on the external surface thereof hydroxyl groups.

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70. The particle of claim 69 wherein the surface hydroxyl groups have been converted to organic functionalities, said functionalities chosen from the group consisting of thiols, amines, and carboxylic acids.

71. The particle of claim 70 wherein the surface hydroxyl groups have been converted to carboxylic acids by mixing the particles of claim 1 with a carboxylic acid in an aqueous methanol/water mixture (10% methanol-80% water) under agitation for period of 5 - 60 minutes.

72. The particle of claim 71 wherein the carboxylic acid is chosen from the group consisting of chloroacetic acid ($ClCH_2COOH$), acetic (CH_3COOH), iodoacetic (ICH_2COOH), 2-chlorobutanoic ($CH_3CH_2CHClCOOH$), 3-chlorobutanoic ($CH_3CHClCH_2COOH$), 4-chlorobutanoic ($ClCH_2CH_2CH_2COOH$), trichloroacetic ($Cl_33CCOOH$), trifluoroacetic (F_3CCOOH), oxalic acid (HO_2CCOOH), malonic acid (HO_2CCH_2COOH), succinic acid ($HO_2C(CH_2)_3COOH$, glutaric acid ($HO_2C(CH_2)_3COOH$, maleic acid (Z-HO_2CCH=$CHCOOH$), and fumaric acid (*E*-HO_2CCH=$CHCOOH$).

73. The particles of claim 70 wherein the surface hydroxyl groups have been converted to amines by mixing the particles of claim 1 and amine-containing organo-silane monomer in a acidified solution at a temperature of 35 -60 °C and washing and dehydrating the particles.

74. The particles of claim 73 wherein the amine-containing organo-silane monomer is chosen from the group consisting of 3-amino- propyltriethoxysilane, 4-aminobutyltriethiethoxysilane, 3-aminopropyl-diisopropyl- ethoxysilane, 3-amino-propylmethyldiethoxylsilane, N-(2-aminoethyl)-3-aminopropylmethyldimethoxysilane, triamino-functional silane ($H_2NCH_2CH_2$-NH-CH_2CH_2-NCH_2CH_2-CH_2-Si-$(OCH_3)_3$, 3-aminopropyltris(trimethylsiloxy)-silane, trimethoxysilylpropyldiethylenetriamine, and 1,3-bis(4-aminobutyl)tetramethyldisiloxane.

75. The particles of claim 70 wherein the surface hydroxyl groups have been converted to thiol groups by incubating the particles of claim 65 in a sodium phosphate buffer with a sufficient excess (10-500 fold) of a solution of a thiolating agent in N, N-dimethylformamide, for about 10 to 100 minutes, and then adding hydroxylamine in a dropwise fashion.

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76. The particles of claim 75 wherein the thiolating agent is chosen from the group consisting of S-acetylmercaptosuccinic anhydride, N-succinimidyl 3-(2-pyridyldithiol)propionate (SPSP), methyl 3-mercaptopropionimidate hydrochloride, 2-iminothiollane.

77. The particles of claim 62, further comprising biological ligands bonded to the organic functionalities, said biological ligands chosen from the group consisting of streptavidin, avidin, oligonucleotides, maleimides, proteins, enzymes, antibodies, antigens, glutaraldehyde, and biotin.

78. The particles of claim 62, further comprising Ni ions bonded to the organic functionalities.

79. The particles of claim 70, further comprising biological ligands bonded to the organic functionalities, said biological ligands chosen from the group consisting of streptavidin, avidin, oligonucleotides, maleimides, proteins, enzymes, antibodies, antigens, glutaraldehyde, and biotin.

80 The particles of claim 70, further comprising Ni ions bonded to the organic functionalities.

81. A method of preparing metallic particles comprising the steps of:

preparing fine silica seeds by hydrolyzing alkyl silicates and silicic acid in an alcohol solutions in the presence of an ammonia catalyst;

oxidatively hydrolyzing iron (II) salt solutions in an alkaline media in the presence of said fine silica seeds, under an inert gas at a pH of from about 7 to about 14, to embed said seeds in a metal oxide core; and

coating said metal oxide cores with a silica protective layer, in a coating process comprising the steps of hydrolyzing an organo silane monomer; condensing the hydrolyzed monomer to oligomers; mixing the cores with the oligomers, under sonication; and drying the oligomer-core mixture to form covalent linkages between the Si atoms of the oligomers and the metal oxide cores.

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82. The method of claim 81 wherein the iron(II) salt is chosen from the group consisting of $FeSO_4$ or $FeCl_2$.

83. A method of preparing metallic particles comprising the steps of:

preparing metal hydrous seeds by hydrolyzing metal ions in an acidic aqueous solution and aging at 500 - 100 °C, and then cooling the solution to room temperature;

oxidatively hydrolyzing iron (II) salt solutions in an alkaline media in the presence of fine silica seeds, under an inert gas such as N_2 at a pH of from about 7 to about 14, to embed said seeds in a metal oxide core;

coating said metal oxide cores with a silica protective layer, said coating process comprising the steps of hydrolyzing an organo silane monomer, condensing the hydrolyzed monomers to oligomers, and mixing the cores with the oligomers, under sonication; and

drying the oligomer-core mixture to form covalent linkages between the Si atoms of the oligomers and the metal oxide cores.

84. The method of claim 83 wherein the iron (II) salt is chosen from the group consisting of $FeSO_4$ or $FeCl_2$.

85. A method of separating a desired substance from a mixture of the desired substance and an undesired substance comprising:

placing magnetic particles having an outer surface in said mixture, allowing said outer surface of said magnetic particles to react with said desired substance to form a particle/substance composite, and removing said particle/substance composite from said mixture by applying a magnetic field to said mixture, said magnetic field being strong enough to overcome attractive forces in said mixture and gravitational forces which, absent the magnetic field, would cause said particle/substance composite to remain in said mixture;

wherein said magnetic particles are superparamagnetic, have a diameter between about 0.03 and about 2.0μ and said magnetic particles comprise magnetic cores completely coated by an inorganic protective layer.

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86. The method of claim 85 further comprising covalently binding biological ligands to the surface of said magnetic particles, wherein said biological ligands increase the affinity of said magnetic particles for said desired substance.

87. The method of claim 86, wherein said particles have chemically active groups on the surface thereof, and wherein said covalent binding process comprises modifying said chemically active groups to chemical functionalities and bonding said biological ligands to said chemical functionalities.

88. The method of claim 85 wherein said desired substance is chosen from the group consisting of antibodies, antigens, DNA, RNA, White Blood Cells, biotin-tagged biological materials, 6x-His tagged biological materials, proteins, and peptides.

89. The method of claim 87 wherein said desired substance is chosen from the group consisting of antibodies, antigens, DNA, RNA, White Blood Cells, biotin-tagged biological materials, 6x-His tagged biological materials, proteins, and peptides.

90. The method of claim the method of claim 87 wherein said inorganic protective layer comprises silica and said chemically active groups comprise hydroxyl groups.

91. The method of claim 90 wherein said chemical functionalities are chosen from the group consisting of amine groups, carboxylic acid groups, and thiol groups.

92. The method of claim 91 wherein said modifying step comprises mixing said magnetic particles with a carboxyl group transferring substance chosenfromthe group consisting of chloroacetic acid ($ClCH_2COOH$), acetic acid (CH_3COOH), iodoacetic acid (ICH_2COOH), 2-chlorobutanoic acid ($CH_3CH_2CHClCOOH$), 3-chlorobutanoic acid ($CH_3CHClCH_2COOH$), 4-chlorobutanoic acid ($ClCH_2CH_2CH_2COOH$), trichloroacetic acid ($Cl_33CCOOH$), trifluoroacetic acid (F_3CCOOH), oxalic acid (HO_2CCOOH), malonic acid (HO_2CCH_2COOH), succinic acid ($HO_2C(CH_2)_3COOH$, glutaric acid ($HO_2C(CH_2)_3COOH$, maleic acid (Z-HO_2CCH=$CHCOOH$), fumaric acid (*E*-HO_2CCH=$CHCOOH$), N-[(3 trimethoxylsilyl)-propyl] ethylenediamine triacetic acid (CH_2-CH_2-CH_2-$Si(OMe)_3$-NH_2-NH_2-(CH_2-$COOH)_3$), 3-trimethylsilylpropionic acid ($Me_3SiCH_2CH_2COOH$), and N-[3-(triethoxysilyl)- propyl] phthalamic acid.

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93. The method of claim 91 wherein said modifying step comprises mixing said magnetic particles with an amine transferring substance chosen from the group consisting of 3-amino- propyltriethoxysilane, 4-aminobutyltriethiethoxysilane, 3-aminopropyl-diisopropyl-ethoxysilane, 3-amino- propylmethyldiethoxylsilane, N-(2-aminoethyl)-3-aminopropylmethyldimethoxysilane, triamino-functional silane ($H_2NCH_2CH_2$-NH-CH_2CH_2-NCH_2CH_2-CH_2-Si-$(OCH_3)_3$, 3-aminopropyltris(trimethylsiloxy)-silane, trimethoxysilylpropyldiethylenetriamine, and 1,3-bis(4-aminobutyl)tetramethyldisiloxane.

94. The method of claim 91 wherein said modifying step comprises mixing said magnetic particles with an thiol group transferring substance chosen from the group consisting of a solution of S-acetylmercaptosuccinic anhydride, 3-(2-pyridyldithiol)propionate (SPSP), methyl 3-mercaptopropionimidate hydrochloride, 2-iminothiollane.

95. The method of claim 87 wherein said biological ligands are chosen from the group consisting of streptavidin, avidin, oligonucleotides, maleimides, proteins, enzymes, antibodies, antigens, glutaraldehyde, and biotin.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09804

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : B22F 3/26, 7/04; B32B 5/16
US CL : 428/553, 555, 558, 570, 403, 404
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 428/553, 555, 558, 570, 403, 404

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,389,482 A (OKANO et al) 14 February 1995 (14-02-95), claims 1-12 & 17.	1-5
X	US 5,599,627 A (AOKI et al) 04 February 1997 (04-02-97), entire document and especifically all claims.	1-12 & 17
X,P	US 5,652,060 A (UCHIDA et al) 29 July 1997 (29-07-97), entire document.	1-12 & 17
A	US 4,271,234 A (SCHONAFINGER et al) 02 June 1981 (02-06-81), entire document.	1-30 & 61
A	US 4,956,220 A (SUEYOSHI et al) 11 September 1990 (11-09-90), entire document.	1-30 & 61
A	US 4,975,214 A (SAKASHITA et al) 04 December 1990 (04-12-90), entire document.	1-30 & 61

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
17 SEPTEMBER 1998

Date of mailing of the international search report
OCT 13 1998

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09804

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,356,712 A (HASHIUCHI et al) 18 October 1994 (18-10-94), entire document.	1-30 & 61
A	US 5,543,069 A (HAYASHI et al) 06 August 1996 (06-08-96), entire document.	1-30 & 61
A,E	US 5,759,435 A (MIYAZONO et al) 02 June 1998 (02-06-98), entire document.	1-30 & 61

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09804

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-30 and 61

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BNSDOCID: <WO 9851435A1 I >

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09804

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-30 and 61, drawn to coated particles wherein the core is a metallic oxide.

Group II, claims 31-60 and 62-82, drawn to coated particles wherein the core is a composite and a method of making thereof.

Group III, claims 83-93, drawn to a method of separating substances.

The inventions listed as Groups I, II and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature of a metallic core coating by an inorganic material... 0.2μ has been shown, see USP 5,389,482 and 5,599,627, not to be a contribution over the prior art. PCT Rule 13.2.

(1) The formation of the coated particles of group II requires a special technical feature, precipitation of seed particles in a mixture in order to form a composite core for the coated particles. This technical feature is not required in the formation of coated particles of group I which contains a homogeneous metallic oxide core.

(2) The method of separating substances of group III requires an application of a magnetic field, a special technical feature that is not required in the formation of coated particles of either group I or II.

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